CORRELATION BETWEEN THE EXPRESSION OF INTEGRINS AND THEIR ROLE IN CANCER PROGRESSION

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Correlation between the expression of integrins and their role in cancer progression

Expression pattern of integrins αvβ3, αvβ5 and α5β1 in clinical and experimental tumour samples

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ABSTRACT

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Title: Correlation between the expression of integrins and their role in cancer progression.

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Keywords: Integrins, Protein expression, Migration, Tumour cells survival, Immunohistochemistry.

The integrins play a crucial role in cancer cell proliferation, migration, differentiation, survival and angiogenesis. It has been shown that integrin expression is positively correlated to cancer dissemination, this suggests targeting selected integrins as an anti-metastatic strategy. The aim of this study is to investigate the effect of novel antagonists of α5β1, αvβ3 and αvβ5 integrins on cancer cell migration, a key process in tumour cell dissemination. Immunohistochemistry was used to evaluate the expression of α5, αv, β3 and β5 integrin subunits in prostate cancer tissues. Furthermore the expression of these integrin subunits in tumour and normal human head and neck tissues was compared. The expression profile of these integrin subunits in established human cancer cell lines was subsequently evaluated using immunodetection methods in cells and xenograft tumour samples. The effect of integrin inhibition on cell migration was then assessed using neutralizing antibodies against αvβ3, αvβ5, and α5β1 integrins in the scratch-wound healing assay. This assay was then used to evaluate the potential of novel small molecule integrin antagonists in preventing tumour cell migration. In H & N tissues, αvβ3, αvβ5 and α5β1 integrins are extensively expressed in tumour tissues but weakly expressed in

normal tissue from the same patient. Further, prostate cancer tissues expressed variable levels of αvβ3, αvβ5 and α5β1 integrins. αvβ3 and αvβ5 integrins were expressed in variable levels in OSC-19, PC-3, DU145, DLD-1, HT-29, HUVEC, MCF-7, MCF-7ADR and M14 human tumour cell lines and in OSC-19, PC-3, HT-29 and MCF-7 xenografts. α5β1 integrin was expressed in all cell lines and xenografts except in MCF-7 cell line and HT-29 cell line and xenograft. Overall, the expression was elevated in xenografts compared to the corresponding cultured cells. Based on the expression profile and ability of cells to migrate, three cell lines (DLD-1 colon, DU145 prostate and OSC-19 HNSCC) were selected as models to further evaluate the potential of novel small molecule integrin antagonists to inhibit cell migration. The cell lines were characterized by using neutralizing antibodies against αvβ3, αvβ5, and α5β1 integrins to determine which of these three integrins were primarily involved in tumour cell migration. In DLD-1 and DU145, blocking αvβ5 and αvβ3 significantly inhibited migration, whilst the migration of OSC-19 was 50% inhibited by a multi-integrin inhibitor combination. Among the antagonists, ICT9055 and ICT9072 significantly decreased DLD-1 cell migration by 70% and 60% respectively while ICT9023, ICT9024, and ICT9026 significantly decreased DU145 cell migration by 60%, 60% and 50% respectively. The findings suggest that single integrin inhibition is not sufficient to prevent cell migration whereas dual or multiple inhibition is more effective. Two novel anti-migratory agents were identified in colon cancer and three in prostate cancer which would warrant further investigation.

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AKT	Protein kinase b
APES	3-aminopropyltriethoxysilane
ATCC	American Type Culture Collection
bFGF	Basic fibroblast growth factor
BSA	Albumin, from bovine serum
BSP	Bone sialoprotein
βTD	β-tail domain
CHAPS	[3-[(3 Cholamidopropyl)- dimethylammonio]-1- propanesulfonate]
CO	Collagen
DAPI	4',6-diamidino-2-phenylindole
DAB	3,3’-diaminobenzidine
DMSO	Dimethyl sulfoxide
DPX	Distyrene-plasticiser-xylene
EC	Endothelial cells
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase

FBS	Foetal Bovine Serum
FC	Flow cytometry
FFPE	Formalin-fixed, paraffin-embedded
Fg	Fibrinogen
FGF2	Fibroblast growth factor-2
FGFR	Fibroblast growth factor receptor
Fn	Fibronectin
GBM	Glioblastoma multiforme
GP	Gleason pattern
GFR	Growth factor receptor
HBSS	Hanks balanced salt solution
HIF-1	Hypoxia-inducible factor-1
H & N	Head and neck
HNSCC	Head and neck squamous cell carcinoma
ICAM	Intercellular adhesion molecule
iC3bi	Complement component
IHC	Immunohistochemistry
IL-8	Interleukin-8
IMDV	Intratumoural microvessel density
IF	Immunofluorescence
KN	Kalinin
LN	Laminin
LSCC	laryngeal squamous cell carcinoma

MAPK	Mitogen-activated protein kinase
MCSs	Multi-cellular spheroids
MEK1	Mitogen-activated protein kinase/extracellular signal-regulated kinase
MIDAS	Metal ion dependant adhesion site
MMP2	Matrix metalloprotease 2
M.O.M	Mouse on mouse
NCI	National Cancer Institute
NF-κB	Nuclear factor-κb
Opn	Osteopontin
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PFS	Progression-free survival
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase a
PSI	Plexin, semaphorins and integrin
RT-PCR	Reverse transcription polymerase chain reaction
S	Stroma
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulfate
SE	Squamous epithelium:
SFK	Src family kinase
TEMED	Tetramethylethylenediamine

TGF-α	Transforming growth factor α
TβRII	TGFβ receptor II
TNF-α	Tumour necrosis factor alpha
TN	Tenascin
TSP	Thrombospondin
TRITC	Tetramethylrhodamine isothiocyanate
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
Vn	Vitronectin
WB	Western blot

1 Chapter 1: Introduction

1.1 Cancer

Cancer is defined as the process of new growth that exceeds and is unrelated to the growth of normal tissues and that continues even when the stimuli evoking the change are removed, thus creating an abnormal mass of tissue (tumour) (Pecorino, 2012). Cancer can be hereditary or sporadic. When multiple members of a family have the same or related cancers, or when there is a germline mutation in the tumour suppressor or proto-oncogenes and the cancer is transmitted from one generation to another, the tumour is classified as inherited. On the other hand, a tumour found in a single, older individual is usually classified as sporadic. 90% of cancer cases are sporadic and the tumours develop as a result of the accumulation of multiple somatic mutations due to environmental factors, such as chemicals or radiation (Boccia et al., 2015, Stewart, 2014).

There are two tumour classifications: behavioural and histogenetic. The histogenetic classification identifies the cells or tissue type from which the tumour originates, for example epithelial, glandular, lymphoid or haemopoietic tissues. This classification is very important when it comes to choosing the best treatment option for the patient. Classification is carried out by a histological examination of the tumour (Underwood and Cross, 2004). Tumours can be classified as benign or malignant, depending on their behaviour. Benign tumours grow slowly, remain localised and are not able to invade the

surrounding tissue or spread to other sites in the body. Conversely, malignant tumours (cancer) grow rapidly and have the ability to invade and destroy adjacent tissue, allowing neoplastic cells to penetrate the walls of the blood vessels and lymphatic channels and so spread to other sites in the body (metastasis) (Pecorino, 2012).

1.1.1 Epidemiology of cancer

Cancer is the second most common cause of death worldwide, with one in three people likely to suffer from cancer during their lifetime (Siegel et al., 2011). In 2012, the estimated number of new cancer cases worldwide was about 14 million and there were 8.2 million cancer-related deaths. The number of new cases is expected to rise by about 70% over the next two decades (WHO, 2015). Carcinoma (malignant epithelial tumours) and lymphoma (malignant lymphoid tumours) are the most common human cancers, while sarcomas (malignant connective tissue tumours) are rare (Pecorino, 2012).

1.1.2 Mechanism of tumour development and metastasis

A complex, multistep process of proliferation, invasion, intravasation, migration, extravasation, colonisation and angiogenesis is necessary for the growth of a primary tumour and metastasis.

Excessive and uncontrolled cellular proliferation or loss of the capacity for apoptosis are considered the main features of cancer. Certain genes, such as proto-oncogenes, are responsible for promoting normal cell growth. Mutant alleles of proto-oncogenes are known as oncogenes, and the activation of

oncogenes such as Bcl-2 leads to abnormal cell growth (Besbes et al., 2015). Moreover, the products of other genes called tumour suppressor genes such as P53, suppress the proliferation stimulating activities of cellular oncogenes and control the rate of cell loss (apoptosis or programmed cell death) (De Oliveira et al., 2015).

Cancer cells are less attached to one another due to changes in the expression of cell adhesion molecules such as cadherin and integrins, which will help the cancer cells to easily break free from their molecular constraints and invade the surrounding tissue (Sawada et al., 2008, Ganguly et al., 2013). Then, these tumour cells adhere to the basement membrane and start to produce, or induce surrounding cells to produce, proteolytic enzymes such as serine proteases and matrix metalloproteases (MMP) to break up the blood or lymph vessel walls (Pecorino, 2012). The tumour cells adhere to the platelets and migrate in the direction of blood flow (Figure 1) (Gay and Felding-Habermann, 2011). When a tumour cell reaches the metastatic site, it escapes from the blood or lymph vessels by adhesion to endothelial cells through various cell adhesion molecules and then passes through the endothelial cells and basement membrane and migrates to the stroma. At the metastatic site, tumour cells interact with the extracellular matrix proteins via cell adhesion molecules such as integrins, which in turn mediate the intracellular signalling that promotes cell proliferation and differentiation. Finally, the tumour cells secrete growth factors and chemokines such as VEGF-A, bFGF, and TNFα, which stimulate quiescent vascular endothelium to enter the cell cycle and create new blood vessels to

provide the tumour cells with the oxygen and nutrients that are necessary for cell proliferation (Avraamides et al., 2008, Hanahan and Weinberg, 2011). The project described in this thesis focuses on the role of integrins in head and neck cancer as well as prostate cancer progression.

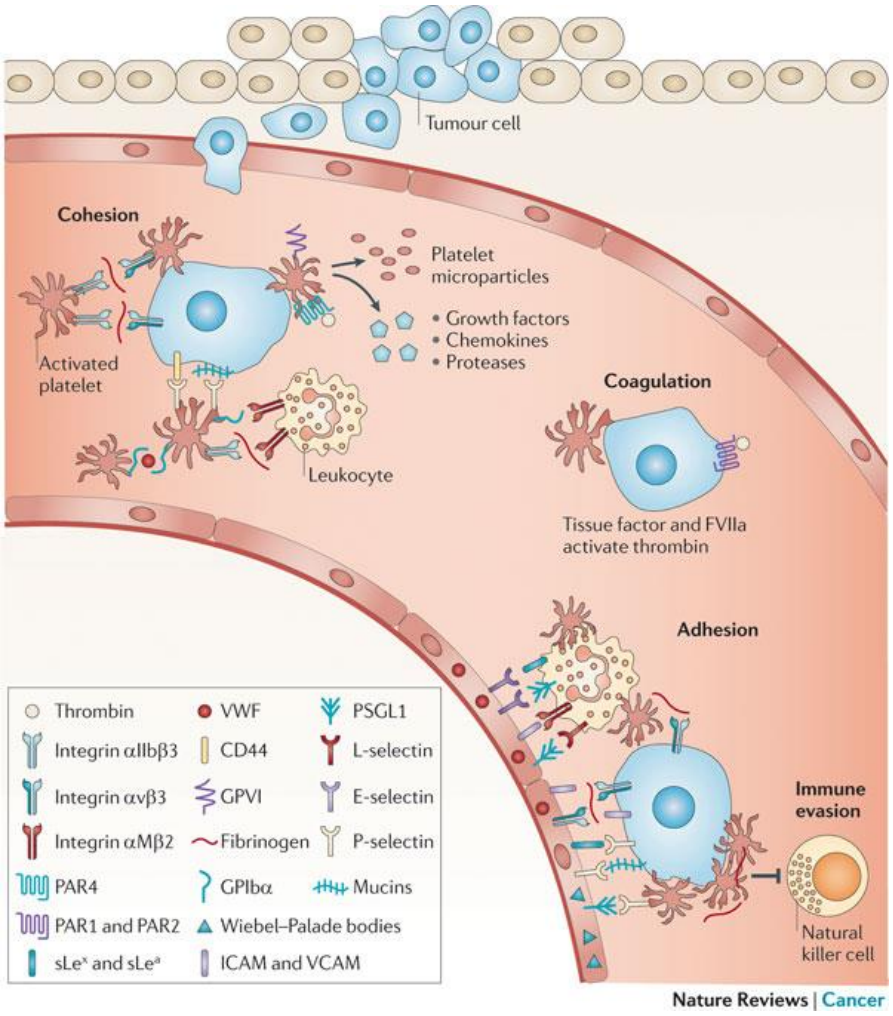


Figure 1 Contribution of platelets to tumour metastasis (Gay and Felding-Habermann, 2011).

1.2 The integrins

Integrins are heterodimeric transmembrane glycoproteins consisting of an α-subunit and a β subunit (Cox et al., 2010). Eighteen different α subunits and eight different β subunits have been reported in vertebrates. These combine to create 24 different heterodimers (Campbell and Humphries, 2011, Hohenester, 2014). There are different types of integrins categorized according to which cell surface, extracellular matrix or inflammatory ligands they bind (Shimaoka et al., 2002). Vertebrates have four receptor subgroups: laminin receptors, leukocyte specific integrins, collagen receptors and RGD receptors which recognise the triplet sequence arginine-glycine-aspartate (RGD) motif found in many ECM proteins such as fibronectin, collagen, and vitronectin, osteoponin and thrombospondin (Figure 2) (Hynes, 2002).

The β1 and αv integrins are the largest subgroups. β1 can be associated with all α subunits except αD, αM, αX, αL, αIIb and αE. The integrin αv subunit can be associated with at least five distinct beta subunits: β1, β3, β5, β6 and β8, whereas the integrin β3 subunit can form heterodimers with only αv and αIIb subunits (Rupp and Little, 2001, Wilder, 2002, Barczyk et al., 2010). These integrins play an important role in cell-cell adhesion. Cells adhere to extracellular matrix proteins and integrins connect the extracellular matrix proteins to the cytoskeleton and active internal signal pathways. Now the integrins have become the target for therapeutic studies aiming to develop drugs to combat thrombosis, inflammation and cancer (Hynes, 2002). This

thesis discusses in detail the structure and function of αvβ5 and α5β1 integrins as well as their roles in normal and diseased states, especially in cancer cells.

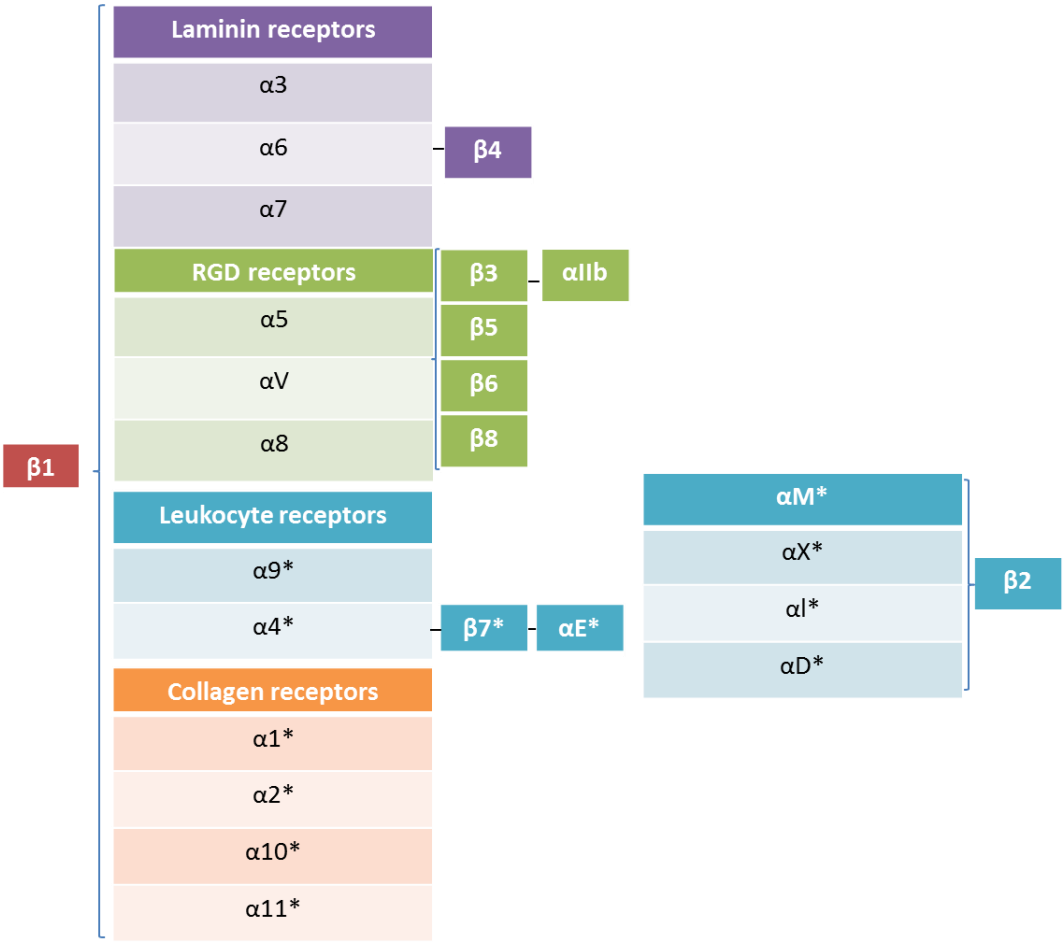


Figure 2 Diagram showing the integrins families.

Half of the α-subunits contain I domains (asterisks) (Shimaoka et al., 2002, Hynes, 2002, Campbell and Humphries, 2011, Liddington, 2014).

1.2.1 Integrin structure

Both α and β subunits are type 1 membrane proteins which have long extracellular domains of >940 and >640 residues respectively, and short intracellular domains (except β4) (Bennett, 2005, Shimaoka et al., 2002). Each α and β integrin subunit has a large extracellular N-terminal globular domain where the ligand binding site is located, a membrane spanning region and a short C-terminal cytoplasmic domain (Figure 3) (Liddington, 2014).

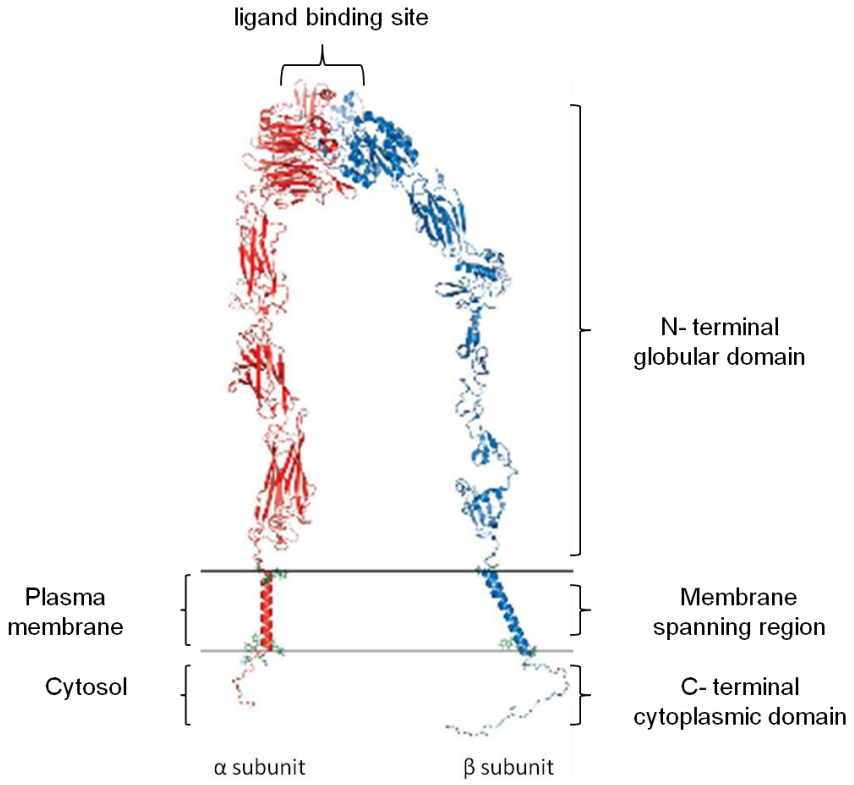


Figure 3 Structure of a non-I-domain Integrin (Lau et al., 2009).

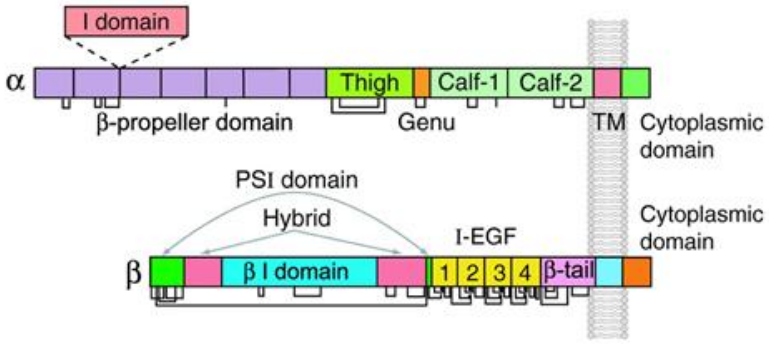


Figure 4 Domains within the primary integrin subunit structures (Luo et al., 2007).

The α chain consists of a seven-bladed β-propeller, followed by one thigh, two calf domains, a transmembrane domain and a short cytoplasmic domain. The region between the thigh and first calf domain is the knee or genu where the head region bends (Figure 4). In nine of the integrins (the collagen receptors and leukocyte specific receptors), there is an inserted domain located between blades 2 and 3 in the β-propeller. This inserted domain is known as the I domain or A domain. The β chain consists of a PSI (plexin, semaphorins and integrin) domain, a hybrid domain, a βI domain, four integrin epidermal growth factor-like domain repeats, β-tail domain (βTD), transmembrane domain and long cytoplasmic domain. The α-subunit β-propeller meets the βI domain at the site where the α head interacts with the β head (Barczyk et al., 2010, Lee et al., 1995a, Shimaoka et al., 2002).

Some integrins (e.g. αLβ2) have two I domains (αI domain and βI domain), whereas other (e.g. αvβ3) have only the βI domain. Both these domains contain

metal ions that interact with ligand, known as the metal-ion dependent adhesion site (MIDAS). The αI domains are the major ligand-binding sites in integrins that contain I domains whereas the βI domain binds ligand in integrins that lack I domains and regulate ligand binding by integrins that contain I domains (Cox et al., 2010, Liddington, 2014, Lee et al., 1995b).

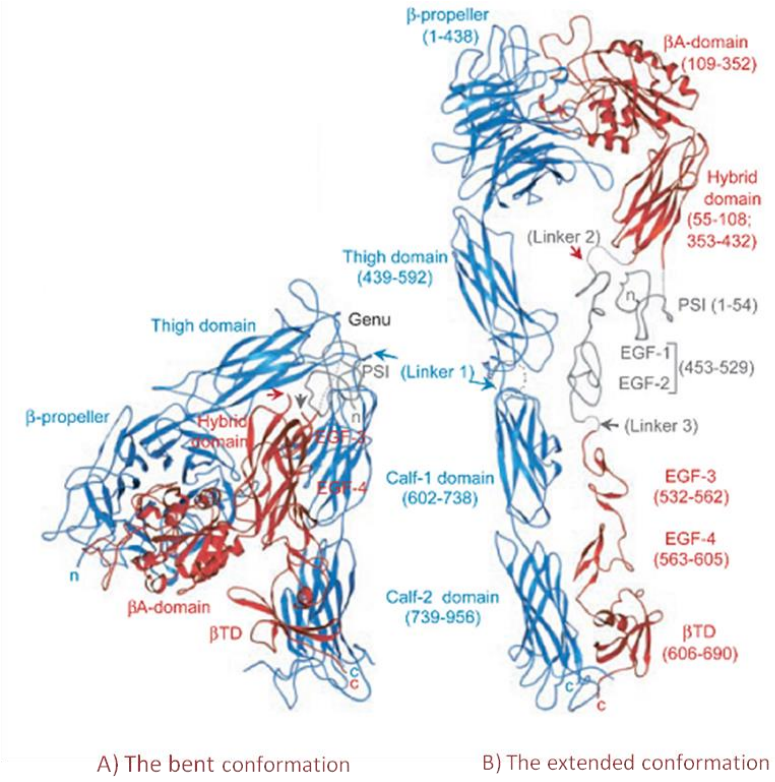


Figure 5 Conformational changes of integrins lacking an I domain.

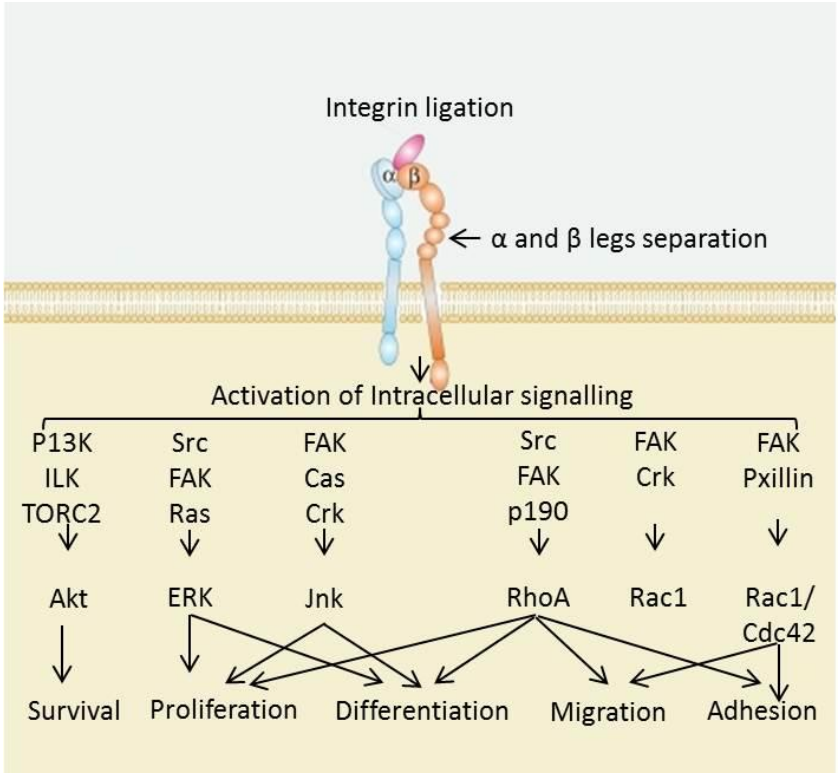
α subunit shown in blue and β subunit in red (Xiong et al., 2001).

Xiong *et al*. found that the crystal structure of a head region of the αvβ3 extracellular region was severely bent over two almost parallel tails. This form is called the bent conformation (Figure 5). The flexed or bent conformation represents the inactive form, whereas the extended conformation of the integrin,

which appeared to be similar to rotary shadowed EM images of αIIbβ3, represents the active form (Xiong et al., 2001),(Xiao et al., 2004). The cell surface normally expresses the integrins in the inactive conformation; however, integrins can be activated by both outside-in (regulating the cell function) and inside-out signalling (regulating cell adhesion) (Millard et al., 2011),(Han et al., 2006).

When the integrins' extracellular domains bind to one of the different ligands that are usually found in the subendothelial matrix, such as fibronectin, vitronectin and collagen, this leads to the creation of outside-in signalling and conformational changes, such as separation of the α and β legs to allow the cytoplasmic tail to interact with intracellular molecules like talin, FAK/c-SRC, small GTPases Ras and Rho, and adaptors like cas/crk and paxillin (Figure 6: A) (Schwartz and Ginsberg, 2002),(Legate et al., 2009), (Cox et al., 2010). Whereas when external stimuli such as selectin ligation or cytokine binding to G-protein coupled receptors stimulates intracellular signalling, this allows cytosolic proteins to bind to the cytoplasmic tail of the integrin. The changes in the tail induce changes in the extracelluar domain, thus regulating the affinity of the integrin for its ECM ligand (inside-out signalling) (Figure 6: B) (Vinogradova et al., 2002),(Takagi et al., 2001),(Xiao et al., 2004),(Cox et al., 2010).

A



B

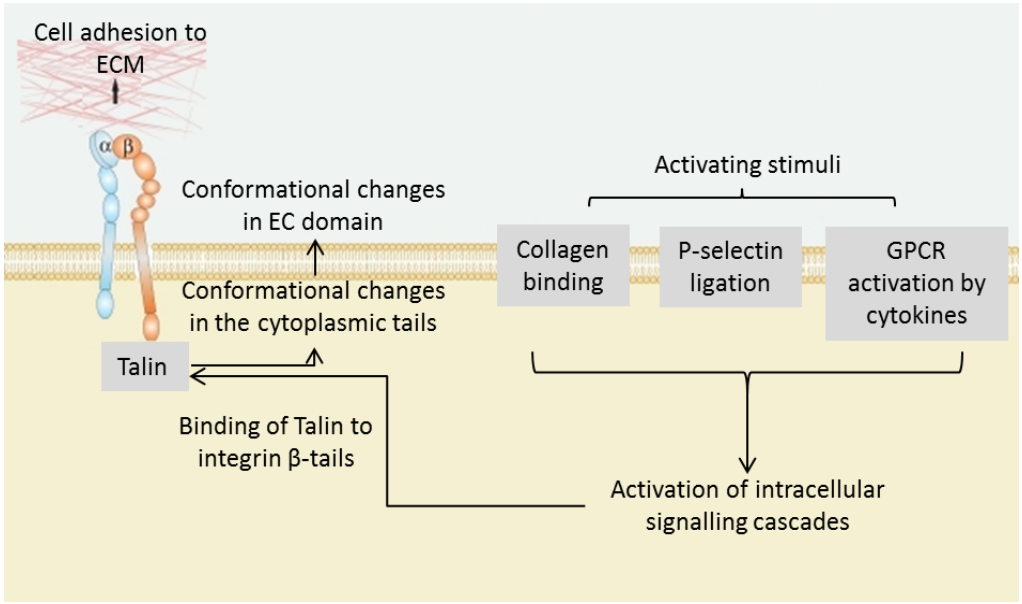


Figure 6 Integrin signalling.

A) Integrin outside-in signalling governs cellular processes. B) Integrin inside-out signalling promotes integrin activation and heightens ligand affinity (Millard et al., 2011).

1.2.2 Integrin functions:

Integrins play an important role in a large number of signalling pathways, controlling various cellular functions such as cell adhesion, tissue integrity maintenance and the promotion of cellular migration, proliferation, survival, division, differentiation and apoptosis. These functions are important in many physiological and pathological processes, such as inflammation, angiogenesis, wound healing, embryonic development, neoplastic transformation and thrombosis (Brooks et al., 1994a, Weis and Cheresh, 2011, Jacobsen et al., 2010, Bloor et al., 2002, Stachurska et al., 2012, Bellucci and Caen, 2002, Seguin et al., 2015). In addition, integrin binding has been recognized as a means of viral entry into cells (Jackson et al., 2002).

1.2.2.1 Function of α5β1, αvβ5 and αvβ3 integrins

There are 11 integrins which contain β1 (αvβ1, α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, α9β1, α10β1 and α11β1). β1 integrins promote cell proliferation, migration, invasion and survival by activating several signalling pathways such as FAK, PI3 kinase and Akt (Hanks et al., 1992),(Meng and Lowell, 1998). The β1 integrins have been characterized in all three developmental layers of the organism (endodermal, ectodermal, and mesenchymal). They are critical in the development and function of the kidney glomerulus (Kanasaki et al., 2008), organogenesis (Potocnik et al., 2000), skin and hair formation (Raghavan et al., 2000), bone and cartilage formation (Aszodi et al., 2003), skeletal muscle and cerebral cortex development

(Schwander et al., 2003),(Belvindrah et al., 2007) and angiogenesis (Mettouchi and Meneguzzi, 2006).

Integrin α5β1 is a major cellular receptor for the extracellular matrix protein fibronectin. It participates in many cell functions including cell adhesion, migration and proliferation (Akiyama et al., 1989). Fibronectin and its receptor α5β1 have an essential role in vascular development during embryogenesis, as early embryonic lethality occurs due to yolk sac and other mesodermal tissue defects in mice in which fibronectin has been deleted (George et al., 1993). Similarly, α5-null embryos display lethal vasculature and cardiac defects (Francis et al., 2002),(Beauvais-Jouneau and Thiery, 1997). It has been shown that α5β1 plays an important role in wound healing; its deficiencies interfere with the migration of epidermal keratinocytes to close the wound (Ongenae et al., 2000, Widgerow, 2013).

The αv subfamily integrins consist of five integrins (αvβ1, αvβ3, αvβ5, αvβ8, αvβ6). All these integrins recognize the arginine-glycine-aspartic acid (RGD) sequence present in certain matrix proteins such as vitronectin, fibronectin, osteopontin, collagens, thrombospondin, fibrinogen and von Willebrand factor (Pytela et al., 1985a, Pytela et al., 1985b). The β5 subunit is present in blastocyst stage embryos and in all embryonic organs such as ependymal layer of the central nervous system, glomeruli of the kidney, epicardial region of the heart, and in the tooth germs suggesting specific functions for this molecule during morphogenetic events in these organs (Bloor et al., 2002),(Yamada et al., 1995). αvβ5 is involved in epithelial-mesenchymal interaction during tooth

development (Salmivirta et al., 1996), regulates lung vascular permeability and pulmonary endothelial barrier function (Su et al., 2007a), and mediates smooth muscle cell migration and neointimal formation in response to injury (Su et al., 2007b), (Dufourcq et al., 2002).

αvβ3 is found on the surface of platelets (expression is low compared to αIIbβ3), active endothelial cells, such as the ductal epithelium of the parotid glands, intestinal epithelium, glomeruli, Bowman's capsules and smooth muscle cells. It is expressed at a high level on osteoclasts, the mid-menstrual cycle endometrium, the placenta, inflammatory sites and invasive tumours (Natali et al., 1997). The cell types that express high levels of αvβ3 include mature bone resorbing osteoclasts and activated macrophages, a small fraction of neutrophils, angiogenic endothelial cells and migrating smooth muscle cells (Wilder, 2002). αvβ3 mediates the adhesion of osteoclast to the osteopontin of bone matrix proteins (Ross et al., 1993). When the integrin interacts with bone matrix protein, this keeps the osteoclast on the bone surface and allows a resorptive cavity to form under the osteoclast. This interaction also promotes osteoclast survival by producing anti-apoptotic signals (Tofteng et al., 2007). αvβ3 antagonism prevents bone resorption *in vitro* by inhibiting osteoclast migration (Nakamura et al., 1999). The role of αvβ3, αvβ5 and α5β1 integrins in angiogenesis and cancer will be discussed in more details later in this report. Some of the integrins' functions are summarized in Table 1.

Integrin	Function	Ligand	References
α1β1	• Binding the osteoclast to collagen. • Angiogenesis. • Lymphangiogenesis.	CO, LN	(Lee et al., 2007),(Hong et al., 2004)
α2β1	• Angiogenesis. • Lymphangiogenesis.	CO, LN, TSP	(Hong et al., 2004)
α3β1	• Neuronal migration. • Controls the expression of MMP2. • Participates in the development of lung and kidney glomeruli. • Keratinocyte migration.	CO, LN-5, FN, TSP	(Anton et al., 1999),(Kubota et al., 1997),(Kreidberg et al., 1996),(Wen et al., 2010)
α4β1	• Promotes angiogenesis. • Regulates embryonic development. • Lymphangiogenesis and tumour metastasis.	VCAM-1, TSP , FN	(Garmy-Susini et al., 2005),(Garmy-Susini et al., 2007),(Sengbusch et al., 2002),(Slack-Davis et al., 2009)

Integrin	Function	Ligand	References
α5β1	• Angiogenesis. • Lymphatic growth. • Cancer metastasis. • Wound healing. • Controls mesodermal formation and function during embryogenesis.	FN, Opn	(Boudreau and Varner, 2004),(Kim et al., 2002),(Francis et al., 2002),(Okazaki et al., 2009),(Strobel and Cannistra, 1999),(Ongenae et al., 2000),(Widgerow, 2013),(Yang et al., 1993)
α6β1	• Skin homeostasis. • Regulates endothelial tube formation.	LN	(Chabut et al., 2003),(Georges-Labouesse et al., 1996)
α7β1	• Cerebral vascularisation. • Muscle development and structural integrity.	LN 1,2,4	(Flintoff-Dye et al., 2005),(Hodges et al., 1997)
α8β1	• Regulates kidney development and other morphogenetic processes. • Regulates spreading, adhesion, growth and survival in neuronal and mesenchymal-derived cell types.	FN, VN and TN	(Denda et al., 1998),(Farias et al., 2005),(Bieritz et al., 2003),(Muller et al., 1995)

Integrin	Function	Ligand	References
α9β1	• Lymphangiogenesis. • Angiogenesis.	TN, Opn, VCAM, FN, CO, LN	(Huang et al., 2000), (Taooka et al., 1999),(Bazigou et al., 2009)
α10β1	• Growth plate morphogenesis and function.	CO-II	(Bengtsson et al., 2005)
α11β1	• Migration/reorganization of fibroblasts throughout the ligament in order to help in tooth eruption.	CO-I	(Popova et al., 2007)
αvβ1	• Cancer cell migration on fibronectin. • Migration of oligodendrocyte precursors.	VN, FG	(Koivisto et al., 2000) ,(Milner et al., 1996)
αvβ3	• Angiogenesis. • Osteoclast function. • Tumour metastasis. • Activation and localisation of MMPs. • Tumour growth and aggressiveness of the tumour cells.	FN, FG, vWF, VN, CO and LN.	(Silva et al., 2008),(Kim et al., 2002),(Sloan et al., 2006),(Hosotani et al., 2002), (Chatterjee et al., 2000)
αvβ5	• Angiogenesis. • Enhances cell migration and liver metastasis of colon carcinoma.	VN, FG, FN.	(Boudreau and Varner, 2004, Kim et al., 2002), (Yoshioka et al., 2010)

Integrin	Function	Ligand	References
αvβ6	• Mediates colon cancer cells proliferation and invasion. • Promotes invasion of squamous carcinoma cells through up-regulation of matrix metalloproteinase-9. • Regulates epithelial remodelling during development, tissue repair and neoplasia. . • Required for wound healing.	FN.	(Cantor et al., 2013),(Thomas et al., 2001),(Breuss et al., 1995),(Jacobsen et al., 2010)
αvβ8	• Brain vessel homeostasis. • Vascularisation in embryo development.	VN, CO and LN.	(Silva et al., 2008),(Zhu et al., 2002)
αIIbβ3	• Platelet aggregation and thrombus formation. • Invasion and metastasis progression of melanoma and prostate cancer.	VN, FG, vWF, TSP, FN.	(Millard et al., 2011),(Cox et al., 2010),(Coller and Shattil, 2008),(Chen et al., 1992),(Trikha et al., 1998a)
αLβ2	• Leukocyte adhesion and migration.	ICAM-1,2,3	(Kinashi, 2007),(Ding et al., 1999)

Integrin	Function	Ligand	References
αMβ2	• Contributes in the maintenance of hematopoietic progenitor cells in the bone marrow. • Participates in the movement of leucocytes from the capillaries to the endothelium.	ICAM, iC3b, FG	(Katayama et al., 2004),(Ding et al., 1999)
αXβ2	• Participates in the adhesion of monocytes to endothelial cells.	FG, ICAM-1, iC3b,	(Keizer et al., 1987)
α4β7	• Recruitment of leukocytes to sites of inflammation Specifically to intestine – Peyer's patches. • Angiogenesis.	FN, VCAM-1	(Kummer and Ginsberg, 2006),(Qin et al., 2006)

Table 1 Integrins functions and their ligands

1.2.3 Integrins and cancer

1.2.3.1 Expression

Studies have shown that integrins on tumour cells are altered quantitatively and qualitatively. Some integrins are expressed at high levels due to continued expression of growth factors and some are absent, while others become phosphorylated or have changes in glycosylation, disturbing the properties of their cytoskeletal and extracellular ligand binding (Fawcett and Harris, 1992) ,(Switala-Jelen et al., 2004),(Mizejewski, 1999). These alterations may help both transformation and tumour progression. Tumour cell expression of integrins is correlated with disease progression in various tumour types as shown in Table 2.

Tumour	Integrins expressed	Correlation with disease	References
Colon	αvβ6	Significantly associated with T stage and TNM stage	(Niu et al., 2014)
Colon	αvβ3	Correlated with poor differentiation and lymph node invasion	(Ibrahim et al., 2013)
Melanoma	αvβ3	Expression restricted to cells within vertical growth phase and metastatic melanomas	(Albelda et al., 1990)
HNSCC	αvβ5	Promotes lymphatic metastasis and angiogenesis.	(Li et al., 2013)
Prostate	αvβ5	Correlated significantly with the Gleason pattern	(Hess et al., 2014)
Prostate	αvβ3	Increased bone metastasis.	(McCabe et al., 2007)
Breast	αvβ5	Increased invasiveness of breast cancer cells.	(Bianchi et al., 2010a)
Breast	αvβ6	Associated with very poor survival and increased metastases to distant sites	(Moore et al., 2014)
Glioblastoma	αvβ3	Associated with a poor prognosis/decreased survival.	(Schittenhelm et al., 2013)
Non-small cell lung carcinoma	α5β1	Associated with decreased survival rates in patients with lymph node-negative tumours.	(Adachi et al., 2000)

Table 2 The correlation between integrin expression and tumour progression

1.2.3.2 Integrins mediate survival and apoptotic pathways.

The microenvironment stimulates the integrins to initiate either a pro-survival pathway or apoptosis. Integrin ligation can initiate a pro-survival pathway by increasing nuclear factor-κB (NF-κB) (Scatena et al., 1998) or PI3K–AKT activity (Aoudjit and Vuori, 2001a), decreasing p53 activation (Bao and Stromblad, 2004) and increasing the expression of the pro-survival molecules BCL-2 and FLIP (Matter and Ruoslahti, 2001, Aoudjit and Vuori, 2001b). Cooperative signalling between growth factor receptors and integrins also protects cells from the intrinsic and extrinsic pathways of apoptosis by activating the Raf pathway (Desgrosellier and Cheresh, 2010). For example, the crosstalk between αvβ3 and fibroblast growth factor receptor (FGFR) and αvβ5 integrin and vascular endothelial growth factor receptor 2 (VEGFR2) prevents endothelial cells from undergoing apoptosis (Hood et al., 2003, Alavi et al., 2003). Unligated integrins can induce apoptosis by activating caspase 8, resulting in apoptotic cell death (Figure 7) (Stupack et al., 2001).

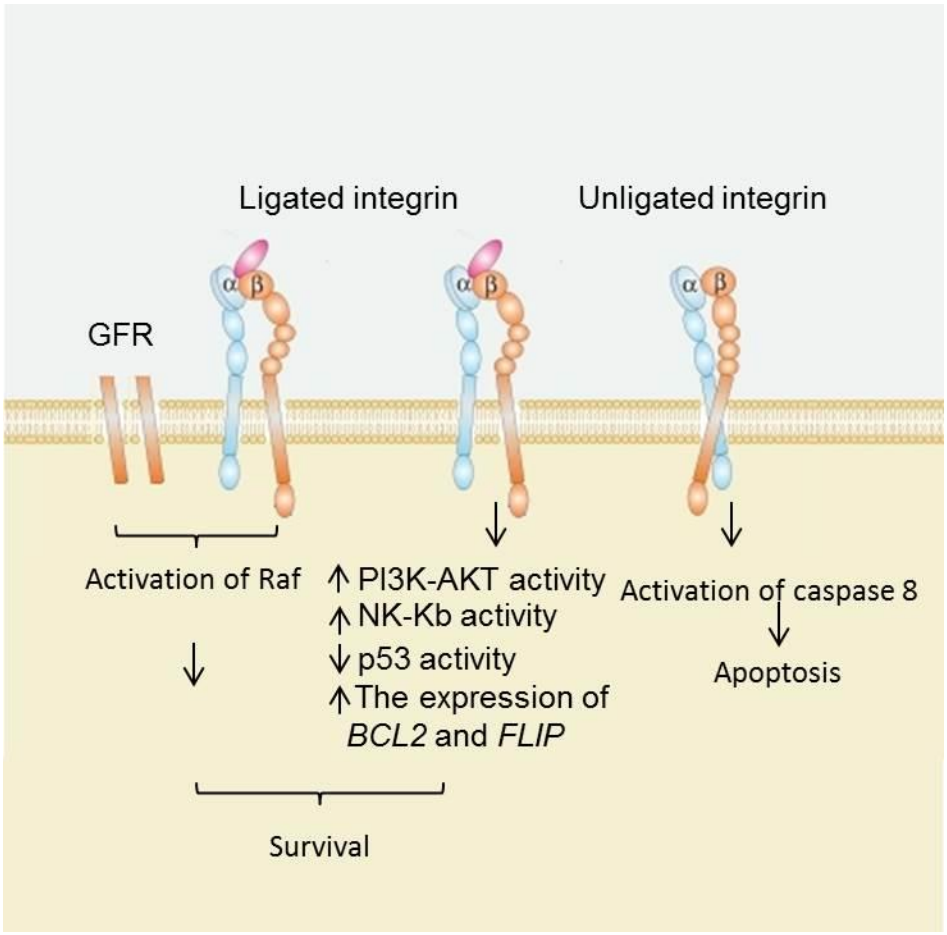


Figure 7 Integrin regulation of cell survival and apoptosis. (Desgrosellier and Cheresh, 2010).

1.2.3.3 Integrins in metastasis

Many reviews have covered detailed of integrins roles in cancer progression for example (Desgrosellier and Cheresh, 2010, Sheldrake and Patterson, 2009, Jin and Varner, 2004, Seguin et al., 2015, Ganguly et al., 2013). The role of integrins in each step of the metastatic process will be discussed below.

1.2.3.3.1 **Invasion from primary tumor site**

The initial step in metastasis is the separation of some cancer cells from the primary tumour mass, which then invade the surrounding tissues as cohesive multicellular units (collective invasion) or individual tumour cells (single cell invasion). There are two proposed methods of single cell invasion: the integrin-dependent mesenchymal invasion program or the integrin-independent Rho/ROCK-dependent amoeboid invasion program (Ganguly et al., 2013, Friedl and Wolf, 2003). In the integrin-dependent mesenchymal invasion program, the cells change shape and convert to a metastatic phenotype during epithelial-mesenchymal transition (EMT), they lose polarity and cell-cell adhesion, and gain migratory and invasive properties (Lamouille et al., 2014). It has been shown that there is crosstalk between TGFβ and integrins such as αvβ3 (Galliher and Schiemann, 2006), αvβ5 (Bianchi et al., 2010b), αvβ6 (Ramos et al., 2009) and α5β1(Maschler et al., 2005) to mediate the EMT process. Furthermore, the ligation of integrins activates a battery of downstream molecules such as focal adhesion kinase (FAK)-Src family kinase (SFK) that mediate cell motility and migration (Wu et al., 2008).

1.2.3.3.2 **Intravasation**

Integrins enable tumour cells to invade surrounding tissue and break up the the blood or lymph vessels wall (Pecorino, 2012) by regulating the localization and activity of matrix-degrading proteases such as matrix metalloproteases (MMPs) and urokinase-type plasminogen activator (uPA) (Brooks et al., 1996). Many integrins such as α5β1, αvβ3 and αvβ6 induce expression and activation of MMP-2, MMP-9 and MMP-3 respectively (Morozevich et al., 2009, Rolli et al., 2003, Ramos et al., 2002, Thomas et al., 2001). Within blood vessels or lymph vessels, platelet integrin αIIbβ3 and tumour receptors such as integrin αvβ3, mediate tumour cell-platelet cohesion which guards tumour cells from immune elimination within the circulatory system. Tumour cells are then passively transferred in the direction of blood flow to distant sites in the body (metastatic sites) (Gay and Felding-Habermann, 2011).

1.2.3.3.3 **Extravasation**

The tumour cells escape from the blood or lymph vessels by adhesion to endothelial cells through various cell adhesion molecules, and then an adhesive bond to endothelial cells is created by integrins. The cells pass through the endothelial cells and basement membrane and migrate to the stroma (Ganguly et al., 2013).

1.2.3.3.4 **Micrometastases**

In the metastatic site, integrins regulate tumour cell proliferation by mediating the expression of key cell cycle proteins and the cyclin-dependent kinase

inhibitor family which control entry into S phase of cell cycle (Fournier et al., 2008).

1.2.3.3.5 **Angiogenesis**

In the metastatic site, new blood vessels must be created to provide the tumour cells with oxygen and nutrients that are necessary for cell proliferation and this process is known as angiogenesis (Pecorino, 2008). When tumour cells proliferate, the local blood supply becomes inadequate, leading to a hypoxic, nutrient-deprived tumour environment. The local hypoxic condition activates hypoxia-inducible factor-1 (HIF-1), resulting in the release of vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF2) and tumour necrosis factor alpha (TNF-α) from proliferating tumour cells and thus leads to activation of integrins which in turn mediate endothelial cell proliferation, differentiation and migration (Figure 8). Many integrins participate in angiogenesis such as α4β1, αvβ3, αvβ5, α4β1, α5β1, and α9β1 (Avraamides et al., 2008). This section will focus primarily on αvβ3, αvβ5 and α5β1 integrins in angiogenesis.

αvβ5 is required for vascular endothelial growth factor (VEGF) or transforming growth factor α (TGF-α) to induce angiogenesis (Friedlander et al., 1995). αvβ5 integrin activates the FAK/Src signalling pathway to stimulate endothelial cell proliferation, differentiation and migration (Hood et al., 2003). Also αvβ5 integrin protects the endothelial cells from intrinsic-mediated apoptosis through activation of Raf and MEK1-dependent pathways (Alavi et al., 2003).

αvβ3 integrin is present on blood vessels in human tumour tissues but not on vessels in normal tissue apart from during growth, wound healing and normal turnover of endometrium (Fabricius et al., 2011, Goodman et al., 2012). Angiogenic growth factors such as basic fibroblast growth factor (bFGF) or tumour necrosis factor α (TNF-α) stimulate the expression of αvβ3 integrin on endothelial cells (Brooks et al., 1994b). In normoxic conditions, cells expressing activated αvβ3 are able to mediate angiogenesis by up-regulating VEGF protein (Lorger et al., 2009).

αvβ3 integrin uses the FAK/Src signalling pathway to stimulate endothelial cell proliferation, differentiation and migration (Eliceiri et al., 1998). Antagonists of αvβ3 induce caspase 8-dependent cell death, so suppressing angiogenesis by inducing endothelial cell apoptosis (Stupack et al., 2001). Antagonists of αvβ3 inhibit angiogenesis and tumour growth in several animal models of cancer (Brooks et al., 1995).

α5β1 integrin mediates endothelial cell migration and survival *in vivo* and *in vitro* by suppressing the activity of protein kinase A (PKA) (Kim et al., 2002). α5β1 is poorly expressed on quiescent endothelium but it is highly expressed in the vasculature of both mouse and human tumours (Kim et al., 2000a). Varner *et al.* found that α5β1 integrin and its ligand fibronectin are up regulated during angiogenesis (Varner et al., 1995). The expression of α5β1 integrin is controlled by many angiogenic factors such as bFGF, IL-8 and TNF-α (Kim et al., 2000a). Ligation of α5β1 has been shown to promote cell proliferation through ERK, Akt and FAK-dependent mechanisms (Lee and Ruoslahti, 2005). α5β1 antagonists,

such as SJ749 and ATN-161 inhibit angiogenesis and thus reduce tumour growth *in vivo* (Kim et al., 2002),(Stoeltzing et al., 2003).

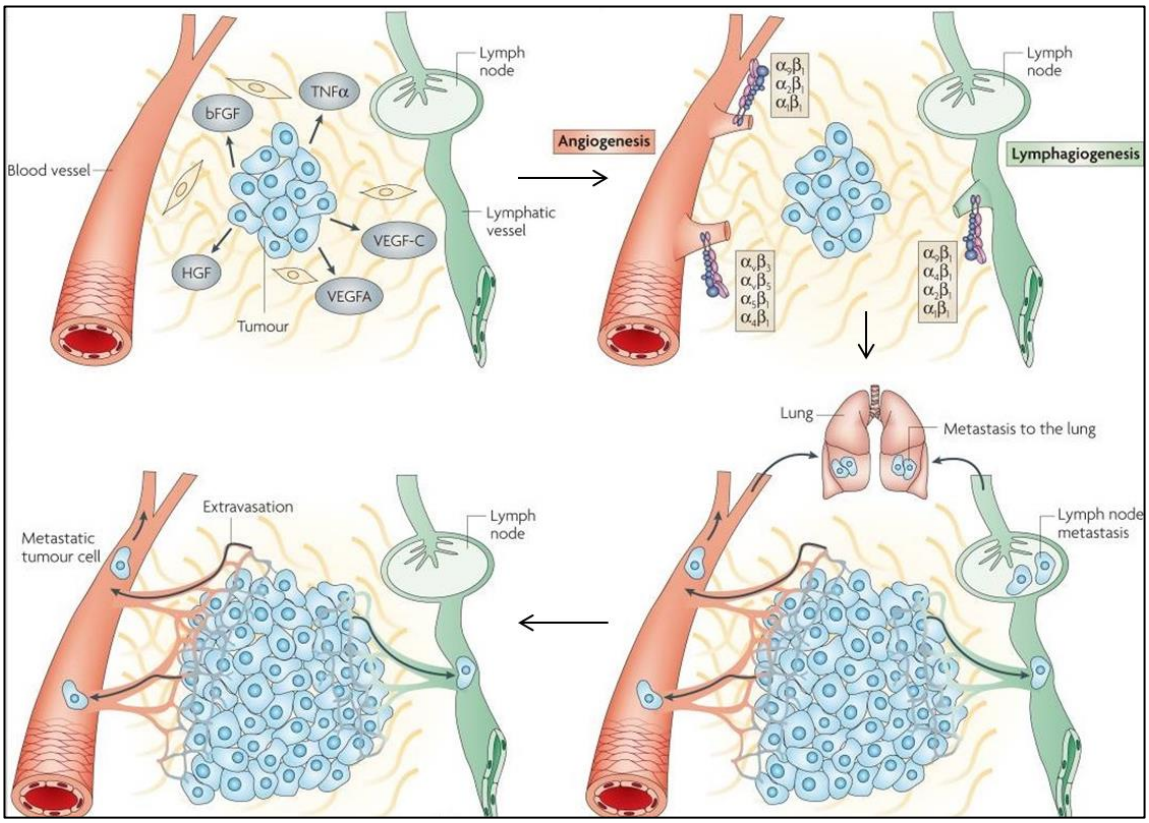


Figure 8 Angiogenesis and lymphangiogenesis mechanisms

Tumour cells stimulate the secretion of growth factors and chemokines which in turn stimulate the expression of α1β1, α2β1, α4β1, α5β1 and αvβ3 integrins on the blood vessels, and α4β1, α9β1, and α1β1 on lymphatic vessels. The integrins mediate endothelial cell migration and survival, leading to creation of new blood vessels which in turn provide nutrients to tumour cells. These new blood and lymphatic vessels promote tumour metastasis to lymph nodes or distant tissues such as lung (Avraamides et al., 2008).

1.2.3.4 Integrins and treatment resistance

Acquired radiation or chemotherapy resistance is a major problem in cancer treatment and it can be developed by many factors such as alteration in the

drug target, initiation of prosurvival pathways or loss of the capacity for apoptosis. Integrin ligation has the ability to initiate a pro-survival pathway and protect the tumour cells from apoptosis, therefore, inhibition of integrin-ligand interaction has become an attractive target for cancer therapy (Seguin et al., 2015, Goswami, 2013). The role of integrins in resistance to radiation or chemotherapy has been identified occurs in multiple types of solid cancers; for example α3β1 integrin is involved in head and neck squamous cell carcinoma cell radioresistance (Steglich et al., 2015, Eke et al., 2012), β1 integrins contribute to breast cancer resistance to trastuzumab and lapatinib (Huang et al., 2011), and αv integrins mediate multicellular radioresistance in human nasopharyngeal carcinoma by activating the SAPK/JNK pathway (Ou et al., 2012). αvβ3 integrin and its ligand osteopontin induce resistance of oral squamous cell carcinoma (OSCC) tissues to 5-FU-based chemoradiotherapy (Nakamura et al., 2015). Interaction between vitronectin and αvβ3 and αvβ5 integrins protects glioma cells from apoptosis induced by chemotherapy (Uhm et al., 1999). Unligated αvβ3 can also mediate resistance; recruiting KRAS and RaIB to the tumour plasma membrane and causing activation of TBK1 and NF-kB pathways that mediate erlotinib resistance (Seguin et al., 2014).

Cancer treatment can also alter the expression of integrins; for example in lung cancer, cisplatin induced a significant alteration in the integrin expression pattern by leading to overexpression of integrin α4, αv, β1, and β5, which led to increased cell motility, while it had no effect on integrin α5, and β3 (Maiuthed and Chanvorachote, 2014). It has been shown that using integrin antagonists in

combination with radiation or chemotherapy increases the treatment sensitivity. Heiduschka *et al*. showed that cilengitide (see section 1.2.4.2.5), in combination with cisplatin, synergistically inhibited cell growth and increased apoptosis due to a decrease in the antiapoptotic protein Bcl-2. They also found that cilengitide in combination with irradiation significantly inhibited colony formation (Heiduschka et al., 2014).

1.2.3.5 Role of RGD-binding integrins in solid tumours

In this section, the role of RGD-binding integrins in cancer progression will be discussed, focusing on two types of cancer; head and neck and prostate cancers because they are the models selected for experimental work in this thesis.

1.2.3.5.1 **Head and neck cancer**

Head and neck cancer is the term used for any malignancies that originate in the oral cavity, oesophagus, pharynx, larynx, paranasal sinuses and nasal cavity (Argiris et al., 2008).. In 2012, about 1,142,000 people were diagnosed with head and neck cancer, while about 776,000 died as a result of the cancer (Ferlay et al., 2015). The most common types of head and neck cancers are oesophageal cancer and oral cancer, which are the 13th and 16th most common cancers in the UK, causing over 7,701 and 2,119 deaths in 2012 respectively (Cancer Research UK, 2012). Squamous cell carcinoma of the mucosal surfaces of the head and neck (SCCHN) is the most common cause and it accounts for about 90% of all such cancers (Walden and Aygun, 2013). Despite the improvement in surgical, radiation and chemotherapy treatments, the five-year survival rate for oral cancers has not improved significantly over the past 30 years, with two-thirds of all patients diagnosed with the advanced stage disease that is characterised by lymph node metastasis (Neville and Day, 2002). Radiation treatment concurrent with chemotherapy has been recognised to improve survival (Adelstein et al., 2003, Bourhis et al., 2012). However, chemoradiotherapy treatment causes severe, long-term side effects and it is

therefore very important to identify targeted therapies and predictive biomarkers in order to improve disease management and minimise the treatment toxicity.

Studies have shown that the expression of epidermal growth factor receptor (EGFR) in head and neck squamous cell carcinoma is associated with advanced stage lymph node metastasis and a poor chance of survival (Mak and William, 2014). It has been found that the targeting of EGFR by antibodies or small molecules improved the treatment of HNSCC patients. Cetuximab which is an antibody targets the cells that express EGFR with either radiation or chemotherapy therapy showed improve in overall survival for patients with HNSCC (Bonner et al., 2006, Vermorken et al., 2008).

Preventing angiogenesis is one of the most rapidly evolving new approaches to cancer therapy. Head and neck cancer is a highly vascularised cancer, which could thus be an ideal candidate for such antiangiogenic strategies. As previously described, integrins play an important role in angiogenesis, particularly the αvβ3, αvβ5 and α5β1 integrins (Avraamides et al., 2008). Targeting these integrins could therefore inhibit angiogenesis and tumour growth.

1.2.3.5.1.1 The αv subfamily integrins (αvβ3, αvβ5, αvβ6, αvβ1, and αvβ8)

The overexpression of the αv integrin subunit in oesophageal, orbital, eyelid, and periorbital squamous cell carcinomas (SCCs) has been shown to be associated with tumour invasion and metastasis (Miller and Veale, 2001). Furthermore, the expression of αv is correlated with the lymphatic metastasis of

laryngeal and hypopharyngeal carcinoma. However, the associated β subunit that plays a major role in SCC aggressiveness has not been identified (Lu et al., 2011). In *in vitro* studies, the expression of αv integrin subunits was evaluated in human nasopharyngeal CNE-2 cells grown as multi-cellular spheroids (MCSs) or as monolayers. MCSs expressed higher levels of the αv integrin subunit than monolayers, and were more resistant to x-ray irradiation. Blocking the αv integrin with an inhibitory peptide was found to increase the radiosensitivity of MCSs *in vitro* and *in vivo* (Ou et al., 2012). Down-regulation of αv-containing integrins by antisense oligonucleotide sequences against the αv subunit inhibited the proliferation of the laryngeal carcinoma cell line (Hep-2 cells) and enhances apoptosis (Lu et al., 2009).

Fabricius et al. compared the expression of αvβ3, αvβ5, and α5β1 integrins between human oral, head, and neck tumour tissues and normal control tissues within the same patients by immunohistochemistry. αvβ3 was extensively expressed in endothelia, and αvβ5 expression was significantly higher in tumour cells and tumour stroma than in normal epithelium (Fabricius et al., 2011). However, the role of the αvβ5 integrin in head and neck cancer is controversial. Certain clinical studies have shown αvβ5 integrin expression to be significantly higher in larynx and tongue SCC cells than in the epithelium cells of normal tissues, and this to be associated with lymphatic metastasis and angiogenesis (Li et al., 2013, Kurokawa et al., 2008). However, Jones *et al*. demonstrated the αvβ5 integrin to be expressed in both normal and tumour cells of oral squamous cell carcinoma, with expression being weak or absent in poorly differentiated

lesions (Jones et al., 1997). The expression of RGD-binding integrins in clinical tissue samples is summarized in Table 3.

RGD-binding integrin	SE	SCC	E	S	Reference
αvβ3	-/+	-/+	+++	+	(Fabricius et al., 2011)
		-			(Jones et al., 1997)
	+	-/+			(Li et al., 2013)
	-/+			+++	(Li et al., 2011)
αvβ5	+	++	++	+++	(Fabricius et al., 2011)
	+	+++			(Li et al., 2013)
	+	++		++	(Jones et al., 1997)
αvβ1	+++	+++			(Li et al., 2013)
αvβ6	-	++	n/a		(Jones et al., 1997)
	++	+++			(Li et al., 2013)
αvβ8	+++	+++			(Li et al., 2013)
αIIbβ3					
α5β1	+	++	+	++	(Fabricius et al., 2011)
	-	+++			(Vitolo et al., 2000)
	-/+	++			(Shinohara et al., 1999)

Table 3 The expression of RGD-binding integrins in squamous cell carcinoma tissues from the head and neck site.

SE; squamous epithelium: SCC squamous cell carcinoma: E; endothelial cells: S; stroma: - negative;-/+ weak; + moderate; ++ strong; +++ very strong; blank not tested.

Moreover, an *in vitro* study showed αvβ5 integrin to be implicated in cancer regression. αvβ5 integrin-deficient cell lines lose the ability of terminal differentiation and grow in an anchorage-independent manner. When an αvβ5 deficient cell line was transfected with the αv subunit, the cells expressed αvβ5 integrins and reversed the *in vitro* malignant phenotype (Jones et al., 1996). In addtion, the transduction of an αv-negative human SCC line (H357 cells) with retroviral vectors encoding αv integrins showed αvβ5 expression to enhance apoptosis (anoikis) by activating caspases 8 and 9 and suppressing AKT activity, (Janes and Watt, 2004).

The strong expression of integrin αvβ3 on endothelial cells in head and neck cancer tissues indicates its role in angiogenesis and this is supported by a recent study conducted by Chuang *et al*. who showed that interaction of αvβ3 with the extracellular matrix-related protein WISP-1 promotes VEGF-A expression which in turn regulates the angiogenesis within the OSCC microenvironment (Chuang et al., 2015). Moreover, it has been shown that activation of αvβ3 to enhance cell migration and invasion (Li et al., 2011). Chuang *et al*. showed that αvβ3 integrin increased the migration of oral squamous cell carcinoma by increasing MMP-3 expression (Chuang et al., 2012).

Several studies have shown oral squamous carcinoma cells express αvβ6, whereas normal oral epithelium does not (Jones et al., 1997, Ramos et al., 1997). Peptide immunolabelling has shown 94.7% of HNSCCs express αvβ6

(Hsiao et al., 2010). Eriksen *et al*. has demonstrated β1, β4, and β6 integrins to be upregulated in carcinomas compared to adjacent mucosa within formalin-fixed, paraffin-embedded pre-irradiation biopsies from 85 patients with HNSCC but the expression was not correlated with tumour or histopathological parameters (Eriksen et al., 2004). αvβ6 expression in primary laryngeal squamous cell carcinoma (LSCC) has also been shown to be higher than in normal epithelium cells, but no significant relationship existed between αvβ6 expression and any clinicopathological features of LSCC (Li et al., 2013).

In vitro studies have suggested αvβ6 is important for cell proliferation and migration. β6 expression has been shown to increase tumour growth *in vitro* and *in vivo* (Ramos et al., 2002), and αvβ6 targeting by an inhibitory peptide significantly inhibited the proliferation of oral squamous cell carcinoma cells (Hsiao et al., 2010). Additionally, it has been shown that the αvβ6 integrin protects SCCs from anoikis by activating an AKT survival signal (Janes and Watt, 2004).

In normal tissues, αvβ6 is expressed at the tips of migrating epithelial sheets during oral mucosal wound healing (Larjava et al., 1996). Forcing the expression of β6 subunits into poorly-invasive human oral SCC9 cell lines by retroviral transduction with full-length β6 cDNA increased SCC-cell migration on fibronectin and invasion through the basement membrane. Blocking of the αvβ6 integrin with an antibody inhibited cell adhesion, migration and growth in a three-dimensional collagen gel, as well as retarding tumour growth *in vivo* (Xue et al., 2001).

αvβ6 has been shown to promote tumour migration by modulating epithelial to mesenchymal transition (EMT). Romes *et al*. found that poorly invasive squamous cell carcinoma SCC9 cells expressing full-length β6 subunits are able to modulate EMT whereas, cells expressing a truncated form of the β6 subunit lacking the C-terminal 11 amino acids are not (Ramos et al., 2009).

Koivisto *et al*. showed that αvβ6 was expressed on 73% of a panel of SCC cell lines screened by flow cytometry. Among these cell lines they selected UT-SCC-14, UT-SCC-7 and UT-SCC-11 cells that have high, moderate and low expression of αvβ6 integrin respectively to evaluate the expression of other integrins and they found that αvβ1 integrin was expressed on the three cell lines but α5β1 integrin expression was higher in UT-SCC-14 than in the other 2 cell lines. These three cell lines were used to study the role of αvβ6, αvβ1 and α5β1 on cell spreading and migration on fibronectin; SCC cells cooperatively used αvβ1, αvβ6, and α5β1 integrins to adhere and migrate on fibronectin (Koivisto et al., 2000).

Recently, Li *et al*. demonstrated the expression of β8 and β1 integrin subunits in primary LSCC cancer to be slightly higher than in normal epithelium, although no significant relationship existed between the expression of these subunits and any clinicopathological features of LSCC (Li et al., 2013).

1.2.3.5.1.2 α5β1 integrin

The role of the β1 integrin subunit in head and neck cancer was studied by Wang *et al*., who used three HNSCC cell lines (M4E, 212LN and PCI-37B), a

xenograft animal model, and tissue samples obtained from patients with HNSCC to demonstrate that β1 expression was significantly higher in metastatic primary tumours than in non-metastatic tumours, and that expression was inversely correlated with overall survival rate of patients. The absence of the β1 integrin reduced lymph node and lung metastasis *in vivo* and matrigel invasion *in vitro* by reducing the levels of active MMP-2 (Wang et al., 2012). Moreover, it has been shown that targeting the β1 integrin with antibodies increases the sensitivity to ionizing radiation and delays the growth of HNSCC cell lines in 3D and xenograft mice (Eke et al., 2012). However, studies looking at β1 without identifying the α subunit bound do not provide definitive evidence for α5β1 as a target.

α5 has been shown to be extensively expressed in biopsy specimens of primary oral squamous cell carcinoma from invasive or metastatic cases. This expression was statistically correlated with tumour invasion and nodal involvement (Shinohara et al., 1999). Oral SCC has been shown to express higher levels of the α5β1 integrin than normal cells (Fabricius et al., 2011) (Vitolo et al., 2000). Also it has been shown that α5β1 integrin is overexpressed in many HNSCC cell lines grown under physiological three-dimensional conditions (Steglich et al., 2014).

1.2.3.5.1.3 αIIbβ3 integrin

αIIbβ3 is primarily expressed by platelets, although expression has also been observed in some tumour types and cell lines, including HSC-3 (Parikka et al., 2006). The clinically used αIIbβ3 antagonist Tirofiban inhibits HSC-3

transmigration. It has also been shown that platelet αIIbβ3 integrin promotes the metastasis of nasopharyngeal carcinoma cells, suggesting interventions affecting platelet activation and tumour-platelet interactions may improve patient survival (Chen et al., 2009).

1.2.3.5.2 **Prostate cancer**

Prostate cancer was the most common cancer in men in the UK in 2011 (Cancer research UK, 2014). In the UK, the estimated number of new prostate cancer cases was about 41,736 in 2011, while there were 10,837 deaths from the cancer in 2012 (Cancer research UK, 2014). Despite the improvement in prostate cancer detection and treatment, treating advanced prostate cancer still a challenge due to the tumour becoming castration resistant and no longer responding to hormone therapy (Amaral et al., 2012). Bone metastasis is common in castration-resistant prostate cancer and it significantly reduces the patient's quality of life due to the severe pain and pathological fractures (Weinfurt et al., 2005). Docetaxel is the most common therapeutic agent for advanced prostate cancer. However, this agent is poorly tolerated (Amaral et al., 2012). Therefore, it is very important to identify molecular target agents that can prevent tumour metastasis to the bone and so improve quality of life. It has been demonstrated that integrins play an important role in the metastasis of prostate cancer cells to bone, as well as in promoting tumour survival, invasion, migration and adhesion to bone vitronectin and osteopontin (Sutherland et al., 2012, Suyin et al., 2013). These factors suggest a potential role for integrin antagonists in this area.

1.2.3.5.2.1 The αv subfamily of integrins (αvβ3, αvβ5, αvβ6, αvβ1, and αvβ8)

The expression and implications of RGD-binding integrins in prostate cancer progression has been previously reviewed in an excellent review by Sutherland (Sutherland et al., 2012). Since then, Hess *et al*. evaluated the expression of a panel of integrins by immunohistochemistry (αvβ3, αvβ5, αvβ6, αvβ8, β3, and αv-pan) in formalin-fixed and paraffin-embedded (FFPE) tissue samples from prostate cancer patients and found that the αvβ6 and αvβ8 integrins were not expressed in tumour cells, and that αvβ3 was expressed in blood vessels only. αvβ5 was almost always expressed in prostate cancer cells in correlation with the Gleason pattern (GP) (Hess et al., 2014). Moreover, Goodman *et al*. confirmed the expression of αvβ3 on vasculature, αvβ5 on tumour cells and the absence of both αvβ6 and αvβ8 integrins from human prostate tissues (Goodman et al., 2012). Lastly, Gorlov *et al*. identified genes that were differentially expressed at different stages of prostate tumourigenesis and found the expression of αv and β5 subunits to be high in prostatic intraepithelial neoplasia compared to normal prostate tissues, although expression of β5 integrins to be negatively correlated with Gleason scores. The α5, β1, β3, and β6 integrins subunits were decreased, and the αIIb, αv, and β5 subunits were increased in the transition from normal prostate tissues to non-metastatic cancer (Gorlov et al., 2009).

The majority of prior studies have focussed on αvβ3, and few studies have examined the role of αvβ5 in prostate cancer. It has been shown that αvβ3 integrin is implicated in the metastasis of prostate cancer cells by mediating cancer cell adhesion to, and migration on, osteopontin and vitronectin in the bone microenvironment (Cooper et al., 2002). Further studies indicate that inactive or constitutively active mutants of αvβ3 integrin prevent tumour growth in bone (McCabe et al., 2007). Moreover, Putz *et al*. evaluated the expression of integrins in nine tumour cell lines established from bone marrow micrometastases of patients with cancer of the prostate (four cell lines), breast (two cell lines), lung (two cell lines), and colon (one cell line) using flow cytometry and they found that α5, αv, β1 and β3 integrin subunits are common characteristics of micrometastatic cells (Putz et al., 1999). In addition, De *et al*. demonstrated the expression of activated αvβ3 and αvβ5 in the tissue sections of metastatic prostate cancer by immunohistochemistry and they reported that the interaction of αvβ3 and αvβ5 integrins with SPARC (secreted protein, acidic and rich in cysteine) which is a component of bone matrix enhances VEGF production, which in turn activate αvβ3 and αvβ5 integrins (De et al., 2003).

Zheng *et al*. report that αvβ3 integrin expressed in primary human prostate cancer cells, yet not in normal prostate epithelial cells. The expression of αvβ3 mediates the adhesion to and migration of PC-3 cells and primary human prostate cancer cells on vitronectin (VN). Transfection of LNCaP with an αvβ3 expression plasmid allowed the previously non-invasive cells to migrate on VN by mediating the FAK- signalling pathway (Zheng et al., 1999). Edlund *et al*.

have shown that LNCaP cells do not express αvβ3, which reduces their migration ability on VN and OPN, whereas these cells' more metastatic sublines (C4, C4-2, and C4-2B) express αvβ3. Blocking αvβ3 by antibodies inhibited migration of the more metastatic cells on VN, and OPN (Edlund et al., 2001). Increased αvβ3 expression in response to CCl2 or 12-LOX has also been shown to promote prostate cancer cell growth, migration and survival (Lin et al., 2013) (Pidgeon et al., 2003).

Bisanz *et al*. demonstrated that knocking-down αv integrin expression in PC-3 cells by liposome-encapsulated human αv-siRNAs inhibited the growth of PC-3 tumours in the skeleton due to enhanced tumour-cell apoptosis in bones (Bisanz et al., 2005). Furthermore, Gordon *et al*. showed the transfection of PC-3 cells with bone sialoprotein (BSP) to result in the increased expression of the αv, β3, and β5 subunits and FAK and ERK phosphorylation, which in turn increased cancer cell invasion and survival (Gordon et al., 2009).

In vitro studies have shown highly invasive human prostate cancer (PC3) and non-invasive (LNCaP) prostate cancer cell lines to not express the β6 integrin (Zheng et al., 1999). Azare *et al*. evaluated by immunohistochemistry the expression of αvβ6 in 40 primary prostate tumours and found that αvβ6 was detected in 9 samples, and its expression was not significantly correlated with clinical stages, Gleason scores, or prostate-specific antigen levels. However, blocking of αvβ6 inhibited tumour cell migration *in vitro* (Azare et al., 2007). Dutta *et al*. found αvβ6 was overexpressed in bone metastases from prostate cancer, indicating it may support dissemination rather than primary tumour

growth (Dutta et al., 2014). Recently, Dutta *et al*. showed that αvβ6 interacts with TGFβ receptor II (TβRII) and consequently activates Smad3 which in turn upregulates MMP2 and enhances cell migration (Dutta et al., 2015). In addition αvβ6 promotes osteolysis by increasing the expression of MMP2 in many prostate cancer cell lines (Dutta et al., 2014). Fedele *et al*. demonstrated that horizontal transfer of αvβ6 *via* exosomes from αvβ6-expressing (PC-3 and RWPE cell lines) to αvβ6-negative recipient cell lines (DU145 and C4-2B) enhanced cell adhesion and migration on LAP-TGFβ (Fedele et al., 2015).

1.2.3.5.2.2 αIIbβ3

Junior *et al*. evaluated the expression of integrins in clinically localized prostate cancer in a tissue microarray to evaluate the association between integrin expression and tumour recurrence following radical prostatectomy in a case-control study. α3β1 and αIIbβ3 expressions were more strongly expressed in recurrent tumours (Pontes-Junior et al., 2010). αIIbβ3 integrin had previously been shown to be expressed in PC-3 and DU145 prostate tumour cells by Western, dot-blotting, and flow cytometry, and in prostate carcinoma tissues by using an *in situ* hybridisation (ISH) technique. Blocking αIIbβ3 inhibited DU-145 cell invasion (Trikha et al., 1996). DU145 cells were found to be more invasive than PC-3 cells due to the expression of αIIbβ3 in focal contact sites allowing it to participate in metastatic progression, whereas PC-3 cells express αIIbβ3 intracellularly (Trikha et al., 1998b). DU145 cell xenografts developed tumours that invaded the surrounding tissue and metastasised to lymph nodes; PC-3

xenografts remained localised to the prostate. Blocking αIIbβ3 prevented lung colonization by DU145 cells in a tail vein injection model (Trikha et al., 1998b).

1.2.3.5.2.3 α5β1

The expression of α5β1 integrin in clinical tissue samples was studied by Nagle *et al*. who reported one case expressed α5β1 integrin from 20 cases of primary prostate cancer (Nagle et al., 1995). Moreover, Chen *et al*. showed that the expression of α5β1 in normal prostate tissues was higher than in prostate cancer tissues. α5β1 expression is positively correlated with tumour's Gleason grade (Chen et al., 2006). Zimmermann *et al*. demonstrated α5β1 to be the primary integrin receptor for fibronectin on human bone marrow stroma, as antibodies against α5, β1, and FN inhibit tumour cell adhesion processes in the bone, (Van der Velde-Zimmermann et al., 1997). Blocking α5β1 significantly inhibited PC-3 cell adhesion to fibronectin and partially inhibited PC-3 cell migration (Stachurska et al., 2012). Lastly, studies have shown in 3D cultures that the α5β1 integrin is the primary integrin that regulates the proliferation of prostate cancer cells by regulating the expression of the sonic hedgehog effector protein, GLI1 (Goel et al., 2010). PHSCN-polylysine dendrimer that targets α5β1 inhibits prostate cancer cells invasion and induction of matrix metalloproteinase 1 (MMP-1) *in vitro*, and inhibits extravasation and lung colony formation *in vivo* (Yao et al., 2010).

1.2.4 Integrin antagonism

All the reported anti-integrin drugs are designed to block integrin-extracellular matrix interaction. Some of the integrin-targeted drugs have made it to the market and some are still in clinical trials. Some integrin antagonists which are in clinical trial are summarised in Table 4. Considerable details regarding integrin antagonism have been reviewed (Goodman and Picard, 2012, Goswami, 2013, Millard et al., 2011, Cox et al., 2010). This section will focus on α5β1, αvβ3 and αvβ5 integrin-targeted drugs.

1.2.4.1 α5β1 integrin antagonists

Due to the role of α5β1 integrin in angiogenesis, a great deal of effort has gone into the development of α5β1 integrin antagonists as treatments for cancer and retinal neovascularisation in age-related macular degeneration. As a result of this effort, there are now several α5β1 integrin antagonists being investigated in pre-clinical and clinical trials, such as:

1.2.4.1.1 **Volociximab (M200)**

Volociximab (M200) is a high-affinity humanized monoclonal antibody against α5β1. It has been developed as an anti-angiogenic agent for the treatment of solid tumours and age-related macular degeneration (Tucker, 2006).

Sawada *et al*. demonstrated that the murine parent antibody of volociximab, IIA1, inhibited the invasion of ovarian tumour cells into matrigel and adhesion to mesothelial cells *in vitro* (Sawada et al., 2008). Volociximab itself inhibited fibronectin binding, HUVEC proliferation, tube formation induced by VEGF

and/or bFGF and induced apoptosis of actively proliferating endothelial cells (Ramakrishnan et al., 2006). *In vivo*, IIA1 decreased metastases and total tumour burden while survivability increased in a mouse SKOV-3ip1 ovarian cancer xenograft model (Sawada et al., 2008). Furthermore, in multiple xenograft models, 339.1 antibody which recapitulates the properties of volociximab inhibited the growth of established tumours by 40-60% and decreased vessel density (Bhaskar et al., 2007).

In a phase I clinical trial, Volociximab could be safely administered at a dosage of 15 mg/kg intravenously weekly. The most common toxicities noted included nausea, fatigue, headache, and arthralgia. One patient with renal cell carcinoma achieved a minor response and 5 patients were confirmed with stable disease (Ricart et al., 2008).

In a phase II clinical trial, weekly infusions of single agent volociximab was well tolerated but without efficacy on patients with platinum-resistant, advanced epithelial ovarian or primary peritoneal cancer (Bell-McGuinn et al., 2011). Volociximab combined with carboplatin and paclitaxel was well-tolerated and led to stable disease in 52% of patients with advanced non-small-cell lung cancer (Besse et al., 2013).

1.2.4.1.2 **ATN-161**

The fibronectin domain containing the sequence PHSRN increases the affinity and specificity of fibronectin binding to α5β1. This PHSRN synergy sequence promotes tumour invasion and angiogenesis. Therefore it has become a target for cancer therapy (Livant et al., 2000),(Stoeltzing et al., 2003),(Wang et al.,

2011),(Cianfrocca et al., 2006). ATN-161 (Ac-PHSCN-NH_2) is a five amino acid peptide targeting αvβ3, αvβ5 and α5β1, derived from PHSRN sequence but replacing the arginine residue with cysteine and derivatising the termini of the peptide (Donate et al., 2008).

ATN-161 inhibited the invasion of DU 145 human and metastatic MATLyLu (MLL) rat prostate carcinoma cells *in vitro* (Livant et al., 2000). ATN-161 in combination with fluorouracil reduced metastasis of colon cancer to the liver (Stoeltzing et al., 2003). Breast cancer cells treated with ATN-161 post radiotherapy showed a significant increase in apoptosis, which in turn led to a reduction in growth of cancer cells in three dimensional culture (Nam et al., 2010). ATN-161 combined with radiotherapy has also been shown to prevent breast cancer metastasis *in vivo* (Khalili et al., 2006). A recent study in ovarian cancer cell line OV-MZ-6 xenograft models showed that targeting α5β1 integrin with ATN-161 in combination with paclitaxel reduced tumour growth by 37.8% compared to controls, while ATN-161 alone had no effect on tumour growth (Kaemmerer et al., 2014).

ATN-161 also possesses anti-angiogenic activity, inhibiting VEGF-induced migration of choroidal endothelial cells and capillary tube formation *in vitro* (Wang et al., 2011). In phase I, ATN-161 was well tolerated at all dose levels. About 1/3 of the patients achieved prolonged stable disease (Cianfrocca et al., 2006). These findings led to preparation of a phase II trial in head and neck cancer in which ATN-161 will be used in combination with radiation and chemotherapy.

A derivative of ATN-161, the PHSCN-polylysine dendrimer (Ac-PHSCNGGK-MAP), has been shown to be more effective in inhibiting prostate cancer cell invasion and induction of matrix metalloproteinase 1 (MMP-1) *in vitro*, and inhibiting extravasation and lung colony formation *in vivo* (Yao et al., 2010). The same results were demonstrated in breast cancer (Yao et al., 2011).

1.2.4.2 αvβ3 and αvβ5 integrin antagonists

1.2.4.2.1 **LM609**

LM609 is a mouse monoclonal antibody that binds αvβ3 integrin. This antibody blocks αvβ3 and inhibits the attachment of endothelial cells to vitronectin, von Willebrand factor (vWF), and fibrinogen (Cheresh and Spiro, 1987). Brook *et al*. demonstrated that LM609 could block angiogenesis in a murine model (Brooks et al., 1995). Subsequently, Mitjans *et al*. showed that LM609 inhibited tumour formation and metastasis in a nude mouse model of human melanoma (Mitjans et al., 1995).

1.2.4.2.2 **Etaracizumab (Abegrin)**

Abegrin is an IgG humanized monoclonal antibody engineered from LM609. In preclinical examination, Abegrin inhibited the adhesion of M21 melanoma cells to fibrinogen (Wu et al., 1998). Moreover, that Abegrin decreased ovarian cancer proliferation and invasion *in vitro* and inhibited the growth of ovarian cancer *in vivo* (Landen et al., 2008).

In phase I studies, stable disease was observed in several patients with renal cell carcinoma. In addition, no serious adverse events and no immune response were observed at the dosages tested (McNeel et al., 2005).

In phase II studies, Hersey *et al*. evaluated the safety and anti-tumour efficacy of Abegrin alone or in combination with dacarbazine in patients with stage IV metastatic melanoma. Etaracizumab with dacarbazine did not appear to be more effective for treatment of metastatic melanoma than single-agent dacarbazine. Consequently, Abegrin is no longer being tested (Hersey et al., 2010).

1.2.4.2.3 **CNTO 95 (Intetumumab)**

CNTO 95 is a fully humanized antibody that recognises αvβ1, αvβ3, αvβ5 and αvβ6. This antibody has anti-tumour and anti-angiogenic properties. In preclinical investigation, CNTO 95 inhibited cell adhesion, migration, proliferation, and invasion of both tumour and endothelial cells *in vitro*. Also, it inhibited human tumour growth in a xenograft model (Trikha et al., 2004). In phase I, no adverse events were observed except infusion reaction which could be controlled with paracetamol. In total 4 of 24 patients achieved stable disease and one patient achieved partial response (Mullamitha et al., 2007). CNTO 95 in combination with standard drugs docetaxel and prednisone was well tolerated in hormone refractory prostate cancer patients (Chu et al. 2007) (Chu et al., 2011). However, CNTO 95 in combination with bevacizumab showed limited clinical activity in patients with refractory solid tumours and the combination had some toxicities (Uronis et al., 2014).

In a phase II trial in metastatic castrate-resistant prostate cancer, intetumumab in combination with docetaxel failed to reach the primary end point of increased progression-free survival (PFS). Median PFS was 7.6 and 11 months for the combination and docetaxel alone respectively; the combination of intetumumab with docetaxel resulted in shorter PFS without additional toxicity among castrate-resistant prostate cancer patients (Heidenreich et al., 2013).

1.2.4.2.4 **S247**

S247 is a small molecule that binds to αvβ3 but also with similar potency to other αv integrins (Shannon et al., 2004). *In vitro*, S247 inhibited cell proliferation, adhesion and migration and also mediated apoptosis (Reinmuth et al., 2003). S247 inhibited endothelial and tumour cell migration and reduced proliferation of endothelial cells but not tumour cells in two highly aggressive orthotopic models of breast cancer metastasis. Continuous infusion of S247 did not reduce the growth of 435/HAL primary tumours and slightly reduced the tumour cells detected in circulating blood but it reduced metastatic tumour burden in the lungs of mice. Administration of the agent for only the first 18 h after tumour cell inoculation into the tail vein resulted in a 68% decrease in the final tumour burden measured by flow cytometry, and an 86% decrease in the tumour-associated gain in lung weight relative to the vehicle-treated group. These results indicate that S247 has potential to prevent both early and late steps of metastasis (Shannon et al., 2004). Further, S247 inhibited the development of colon cancer liver metastasis *in vivo* but did not inhibit the growth of the primary tumour.

Radiation increases the expression of $\alpha_v\beta_3$ in endothelial cells and resulted in phosphorylated Akt, which increases the pro-survival signaling but in combination with S247, the radiation-induced Akt phosphorylation is inhibited and the radiosensitivity of endothelial cells is enhanced. *In vivo*, combination of radiation with S247 resulted in a significantly greater delay in tumour growth, reduced tumour cellular proliferation and decreased tumour vessel growth than that produced by monotherapy (Abdollahi et al., 2005).

1.2.4.2.5 **Cilengitide (EMD 121974)**

Cilengitide is a cyclic pentapeptide containing the RGD sequence. It recognises αvβ3 and αvβ5. Cilengitide is proposed to inhibit tumour growth by two mechanisms of action: first, cilengitide inhibits angiogenesis by preventing the interaction between endothelial cell integrins and their ligands, thereby promoting apoptosis of tumour associated endothelial cells; second, it may reduce tumour growth by inhibiting the interaction between tumour cells expressing αvβ3 or αvβ5 and the extracellular matrix, which then promotes apoptosis of these cells (Reardon and Cheresh, 2011).

Initial preclinical studies showed that cilengitide inhibited the adhesion, growth, and proliferation of tumour cells in xenografts. Xenograft studies have demonstrated the activity of cilengitide as a single agent against brain tumours, in combination with radiation in breast cancer, and with temozolomide in malignant melanoma (Reardon and Cheresh, 2011),(Burke et al., 2002),(Tentori et al., 2008). MacDonald *et al*. implanted human brain tumour cell lines DAOY (medulloblastoma) and U87 MG (glioblastoma) into either the forebrain

(orthotopic) or the subcutis (heterotopic) of nude mice, and systemically treated these animals with cilengitide to observe the difference in microenvironment in the growth of tumours and response to the drug. In the brain, cilengitide showed good activity, blocking the growth of tumour cells completely, whereas in skin, the drug had no effect (MacDonald et al., 2001). Recently, Ruffini *et al*. showed that cilengitide inhibits melanoma cells invasion, vasculogenic mimicry and secretion of VEGF-A and metalloproteinase 9 (Ruffini et al., 2014).

Huber *et al*. demonstrated cilengitide with cetuximab to have a synergistic antitumor activity effect on A431 epidermoid carcinoma and U87MG glioblastoma cell xenografts (Vermorken et al., 2014). Heiduschka *et al*. evaluated the effect of cilengitide as a single agent or in combination with chemotherapy (cisplatin) or irradiation on the proliferation and colony formation of three HNSCC cell lines (SCC25, CAL27 and FaDu) and found that cilengitide, in combination with cisplatin, synergistically inhibited cell growth and increased apoptosis due to a decrease in the antiapoptotic protein Bcl-2, which is associated with chemo-resistance in various malignancies. They also found that cilengitide in combination with irradiation significantly inhibited colony formation (Heiduschka et al., 2014).

In a single patient case study, cilengitide with gemcitabine inhibited the growth of a highly vascularized head and neck tumour and prolonged life for more than one year encouraging further investigation in HNSCC (Raguse et al., 2004). In the first phase I study the safety and toxicity of cilengitide in dosages between 30-1600 mg/m^2 twice weekly among adult patients with recurrent solid tumours

were evaluated and no dose limiting toxicities observed (Eskens et al., 2003). Cilengitide was subsequently evaluated in phase I trials in adults with recurrent malignant glioma in dosages of 120-2400 mg/m^2 twice weekly. Several dose limiting toxicities were observed (in 6/51 patients), thrombosis, joint and bone pain, thrombocytopenia, anorexia, hypoglycaemia, and hyponatraemia, but no MTD was found. Two patients demonstrated a complete response, three patients had partial responses, and four patients exhibited stable disease (Nabors et al., 2007).

In phase II, Reardon *et al*. assessed the activity and safety of cilengitide in glioblastoma multiforme (GBM) patients at first recurrence, who received dosing of either 500 or 2000 mg infusion of cilengitide twice weekly for up to 48 weeks. Greater antitumour response was observed at the 2000 mg dosage (Reardon et al., 2008).

Several studies have evaluated the effects of cilengitide when combined with treatment regimens for pancreatic cancer and GBM. Treatment of advanced stage pancreatic cancer patients with a combination of cilengitide with gemcitibine failed to demonstrate a difference in outcome compared to treatment with gemcitabine alone (Friess et al., 2006). Cilengitide has been evaluated on newly diagnosed GBM patients in combination with radiation and chemotherapy. The one year overall survival was 67% for patients on cilengitide combined with radiation and chemotherapy and 62% for patients on radiation and chemotherapy alone (Stupp et al., 2010). A Phase II trial in patients with head and neck squamous cell carcinoma showed that cilengitide in combination with cisplatin, 5-fluorouracil, and cetuximab did not improve progression-free

survival (Vermorken et al., 2014). Moreover, in a phase III trial of cilengitide combined with standard treatment in MGMT-promoter methylated glioblastoma, cilengitide did not improve the overall survival of patients with glioblastoma; median overall survival was 26.3 months in the cilengitide group and in the control group (Stupp et al., 2014). Consequently, the clinical development of cilengitide has been discontinued.

The trial investigators suggested that the failure of cilengitide in head and neck cancer is because HNSCC cells (in contrast to tumour endothelium) tend to express low levels of αvβ3, the integrin most tightly bound by cilengitide (Fabricius et al., 2011). Tumour expression of related integrins not targeted by cilengitide may also provide a mechanism for drug resistance and therapeutic failure. Moreover, Becker *et al*. showed that cilengitide was not distributed from the plasma to other compartments, and was mainly just excreted by the kidney. Its short half-life means tumours are not exposed to therapeutic concentrations for much of the time and may promote tumour growth; poor pharmacokinetics has ultimately led to its development being discontinued (Becker et al., 2015, Stupp et al., 2014).

Drug name	Target	Type of drug	Disease indication	Status	References
Volociximab	α5β1	Antibody	Untreated non-small-cell lung cancer	Phase II	(Besse et al., 2013)
ATN-161	α5β1, αvβ3 and αvβ5	Non-RGD-based peptide	Recurrent intracranial malignant glioma	Phase II	(Cianfrocca et al., 2006).
JSM6427	α5β1	Small molecule	Age-related macular degeneration	Phase I	(ClinicalTrials.gov)
Etaracizumab	αvβ3	Antibody	Melanoma Cancer Rheumatoid arthritis Plaque psoriasis	Phase II	(Hersey et al., 2010). (Wu et al., 1998),(McNeel et al., 2005),(Hersey et al., 2010),(Brooks et al., 1995)
CNTO 95	αv	Antibody	Metastatic castration resistant prostate cancer	Phase II	(Heidenreich et al., 2013)
Cilengitide	αvβ3 and αvβ5	Cyclic RGD pentapeptide	Glioblastoma Melanoma	Phase III	(Stupp et al., 2014)
DI17E6	αv	Antibody	Castration resistant prostate cancer and bone metastases	Phase II	(Wirth et al., 2012)
MK-0429	αvβ3	Small molecule.	Metastatic bone disease in patients with hormone-refractory	Phase I	(Rosenthal et al., 2010)

			prostate cancer.		
Natalizumab	α4β1 and α4β7	Antibody	Multiple sclerosis and Crohn's disease	Approved	(Miller et al., 2003)
Abciximab	αIIbβ3 αvβ3 αmβ2	Antibody	Percutaneous coronary intervention. Acute Coronary Syndromes. Myocardial Infarction.	Approved	(Tam et al., 1998),(Mager et al., 2003),(Mazzaferri and Young, 2008),(Yeung and Holinstat, 2012)
Eptifibatide	αIIbβ3	A synthetic cyclic heptapeptide	Thrombosis	Approved	(Dennis et al., 1990),(Yeung and Holinstat, 2012)
Tirofiban	αIIbβ3	Small molecule	Thrombosis	Approved	(Hartman et al., 1992),(Cannon et al., 2001)

Table 4 Integrin antagonists in clinical use and clinical trials

1.3 Overall aims and objectives

The aim of this study was to evaluate the effect of potential novel small molecule RGD integrins antagonists on the inhibition of human tumour cell migration using 2D wound healing assays (scratch assays). This was achieved by:

- Evaluating the targets (αvβ3, αvβ5 and α5β1 integrins) in clinical tumour samples.
- Detecting the expression of αvβ3, αvβ5 and α5β1 integrins in human xenograft tissue as well as in human tumour cell lines.
- Developing models that can be used for both the *in vitro* and *in vivo* evaluation of novel small molecule integrin antagonists

2 Chapter 2: Characterisation of integrin expression in clinical head and neck and prostate tumour samples and investigation of correlation between integrin expression and cancer aggressiveness

2.1 INTRODUCTION

Extracellular matrix (ECM) proteins have been shown to play a very important role in cancer progression; these proteins keep the cells linked to matrix elements and to the basal membrane, and also contribute to the matrix-cell signalling cascades that transmit from the ECM to the cells through integrin molecules. Changes in the interaction between ECM proteins and integrins lead to the activation of cytokines, growth factors and intracellular adaptor molecules, which have an effect on tumour behaviour, especially with respect to proliferation, survival, signal transduction and migration (Hu and Luo, 2013, Pecorino, 2012, Hehlgans et al., 2007, Rathinam and Alahari, 2010). In order to understand the mechanisms involved in these processes and identify molecular targets for therapy, the altered integrins and their correlations with clinical and pathological stages must first be demonstrated to be relevant to the tumour.

Determining the integrin distribution in human samples is crucial because the benefit of drugs that target integrins is correlated with integrin expression (Sheldrake and Patterson, 2014). The previous studies detected the expression of integrins in cell lines and only on fresh frozen tissues originating from human

or human tumour cell xenografts. This limitation is due to the lack of monoclonal antibodies that label integrins in formalin-fixed, paraffin-embedded tissues (FFPE) which are the routine diagnostic samples in surgical pathology. This problem obstructs the progress of drugs that target αv integrins, by confounding the identification of tumour types and patient subgroups which would benefit from treatment. More recently, matched rabbit monoclonal antibodies against integrins that are useable with native and FFPE human tissues have been produced but unfortunately these are not commercially available (Goodman et al., 2012). These novel antibodies have led to numerous studies to evaluate the expression of integrins in (FFPE) human tissue, such as colorectal (Denadai et al., 2013, Ha et al., 2014), prostate (Hess et al., 2014), lung (Boger et al., 2014), brain (Schittenhelm et al., 2013) and head and neck (Fabricius et al., 2011).

The current therapies in head and neck cancer and prostate cancer are not totally successful; therefore new therapeutic strategies are required. Head and neck cancer is a highly vascularised cancer that expresses integrins. Studies have shown that antiangiogenic drugs prevent cancer progression and integrin antagonists have good potential in this area (Avraamides et al., 2008, Weis and Cheresh, 2011). In prostate cancer, integrins have been implicated in growth, spread and tumour growth in bone. Many reviews have heavily emphasized the importance of RGD integrins as major pharmaceutical targets in prostate cancer (Sutherland et al., 2012, Goswami, 2013). Therefore, this study investigated the expression of αvβ3, αvβ5 and α5β1 integrins on normal and head and neck cancer, and prostate cancer tissues obtained in Bradford.

2.2 AIMS AND OBJECTIVES

The aim of the research described in this chapter was to characterise integrin expression and investigate any a correlation between expression and tumour progression. This was achieved through the following objectives:

- Characterise the expression of αv, β5, αvβ3 and α5β1 integrins in head and neck tumours and normal tissues from the same patient
- Characterise the expression of αv, β5, αvβ3 and α5β1 integrins in prostate cancer tissues
- Use statistical analysis to investigate if there is a relationship between expression and tumour aggressiveness.

2.3 MATERIALS AND METHODS

2.3.1 Patients and tissue samples

This project was approved by Ethical Tissue Licensed Research Tissue Bank (ISAC Reference: Application/13/050) University of Bradford in Bradford, UK, which is authorised to provide ethical approval from the NHS Leeds (East) Research Ethics Committee (07/H1306/98). The clinical characteristics of the patients are summarized in Table 5 and Table 6. Paired samples, consisting of tumour tissues and control non-cancerous tissues obtained from the same patients with head and neck cancer [the control normal tissues were collected from the area beyond the cut edge or the surgical margin] (n = 10) or prostate cancer (n = 9) were obtained *via* surgery and were snap frozen in liquid nitrogen and stored at −80°C until sectioning. The samples of head and neck tissue were collected from patients whose ages ranged between 57 and 90 years; samples of prostate cancer tissue were collected from patients whose ages ranged between 48 and 65 years. For head and neck cancers, the tumour stage was classified according to TNM classification, in which T indicates the size of the primary tumour and any tissues of the oral cavity or oropharynx to which it has spread, N describes the extent of the spread to nearby (regional) lymph nodes, and M indicates whether the cancer has spread (metastasized) to other organs of the body (Table 5 and Table 6)(Medscape Drugs & Diseases 2013). Prostate cancers were classified by TNM classification and Gleason score (Cancer research UK, 2014), although full data was not available on all patients.

Before cutting sections, tissue blocks were equilibrated to the cryostat temperature of −20 ºC for 24 hours. Sections (5-µm thickness sections) were

cut from the tissue blocks using a microtome cryostat (Leica CM18604v, Leica Microsystems, Nussloch, Germany). Sections were placed on slides coated with 3-aminopropyltriethoxysilane (APES) and left at room temperature (approximately 22 °C) until the sections adhered to the slides. Slides were stored at −80 °C until stained.

No	Localization	Gender	Age	TNM stage	stage
2673	SCC of floor of mouth	Male	57	pT2, pN2c, pMx	4
3085	SCC of left tongue	Female		pT2 pN2b pMx	4
3091	SCC buccal mucosa	Female	83	pT1 pN2b (both)	4
3092	SCC tongue	Female	90	pT4a, pN2b, pMx	4
3554	SCC of mouth (left)	Male	58	pT4a, pN1	4
3555	Verrucous carcinoma of maxilla	Male	72	pT4a, pN0	4
3824	SCC right tonsil	Male	68	pT4a/4b, pN1, pMx	4
3545	Invasive SCC of mouth	Male	59	pT3, pN1, pMx	3
3340	SCC maxillary sinus	Male	59	pT3, pN0, pMx	3
3325	SCC left retromolar region	Female	80	pT4, pN0	4

Table 5 Clinical characteristics of patients with head and neck cancer

No	Localization	Gender	Age	TNM stage
3064	Adeocarcinoma	M	49	Gleason 6 (3+4). pT2, Nx, Mx
3586	Acinar type adenocarcinoma	M	57	Gleason sum 7 pT2C N0 Mx
2759	Adenocarcinoma	M	51	Gleason 6 (3+3). pT2, Nx, Mx
3515	Adenocarcinoma	M	65	Gleason sum 6 (3+3) pT3a No Mx
3592	Microacinar adenocarcinoma	M	48	Gleason 7 (3+4) pT2a No Mx
6200	Prostatic adenocarcinoma	M	65	pT2c
6201	Prostatic adenocarcinoma	M	60	Gleason 7 (4+3), pT3b
6197	Adenocarcinoma	M		Gleason 6 (3+3) pT3a
2758	Adenocarcinoma	n/a	n/a	n/a

Table 6 Clinical characteristics of patients with prostate cancer

2.3.2 Haematoxylin and eosin staining of frozen tissue

Frozen tissue sections were fixed for 10 minutes with acetone at room temperature and then rehydrated for 10 minutes in phosphate buffered saline (PBS). Fixed tissues were immersed in Harris's haematoxylin solution for 10 minutes before they were washed with running tap water. The tissues were soaked in an alcohol/acid solution (0.25% HCl in 70% ethanol) for 10 seconds and then blued in Scott's Tap Water (20 g sodium bicarbonate and 3.5 g magnesium sulphate in 1 L of distilled water) for 2 minutes. Tissues were counterstained with 1% aqueous eosin for 1 minute and rinsed with running tap water. Sections were then dehydrated for 2 minutes in 100% ethanol, cleared

for 2 minutes with 50% xylene/ethanol and 100% xylene and coverslip-mounted with a distyrene-plasticiser-xylene (DPX) medium (VWR International Ltd). Slides were stored at room temperature until stained.

2.3.3 Immunohistochemistry of frozen sections

Frozen tissue sections were fixed 10 minutes in acetone at room temperature and then rehydrated for 10 minutes in PBS (Sigma-Aldrich). The sections on the slides were circled with a wax pen and then blocked by adding 300 µl blocking agents 5% BSA (Sigma-Aldrich), 10% goat serum (for PIF6 and Q-20) or horse serum (Millipore) (for LM609 and JBS5) for 1 hour at room temperature. Excess blocking was removed and 100 µl of diluted primary antibody was added and incubated 1 hour at room temperature. Primary antibodies were withheld from negative controls. The tissues were washed three times for 10 minutes in PBS and then incubated in 100 µl of diluted secondary antibody (1:200) for 30 minutes at room temperature. Tissues were washed three times for 10 minutes in PBS. Tissues were incubated with ABC peroxidase labelled streptavidin solution for 30 minutes at room temperature. The ABC reagent was washed off, and the tissues were washed three times for 10 min in PBS and then incubated 5 minutes in 3,3'-diaminobenzidine (DAB Peroxidase (HRP) Substrate Kit, Vector laboratories), counterstained with haematoxylin for 20 seconds and blued in Scott's Tap Water for 2 minutes. Finally, tissue sections were dehydrated, cleared and mounted as described above (Section 2.3.2). The steps that were followed to optimize the antibodies are shown in Figure 9. The primary and secondary antibodies used in immunohistochemistry are

summarized in Table 7. To identify the squamous epithelium cells in the frozen sections, mouse monoclonal anti-pan cytokeratin were used, without scoring the expression.

2.3.3.1 Evaluation of integrin expression

The immunohistochemical labelling was scored semi-quantitatively in a blinded process by a senior clinical pathologist (Dr Philip Batman). Immunohistochemical staining intensity was scored as (0) for negative staining, as (1) for weak staining, as (2) for moderate staining, as (3) for strong staining and as (4) for very strong staining (Figure 10).

2.3.3.2 Statistical analysis

The analysis was carried out by Dr Andrew Scally using Stata v13.1. The correlations between integrin expression and clinical characteristics were examined using Fisher's exact t-test. Due to the limitations of the small sample size (head and neck sample = 10; prostate sample = 9), a very large observable effect would be required to show statistical significance. For these preliminary investigations, a P value of < 0.2-0.4 was used as an indicator of potential trends which could be further investigated. A qualitative inspection of the data was performed to tentatively confirm the existence of trends. Additional studies with a larger sample size should, however, be performed to verify the significance of any trend. Differences in integrin expression between paired tissue types from individual patients were examined using the Wilcoxon signed-rank test.

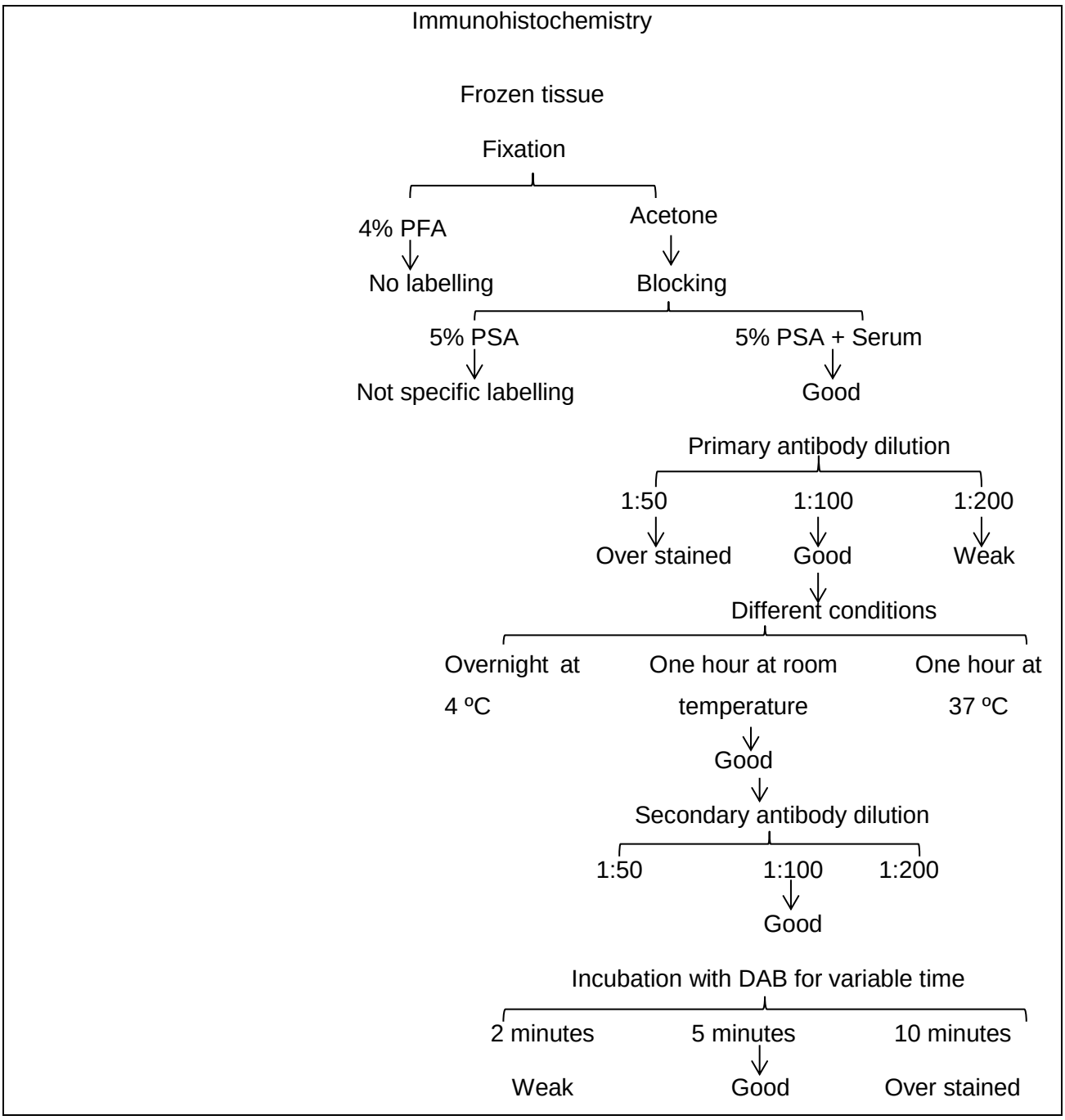


Figure 9 The steps which were followed to optimize the antibodies in immunohistochemistry.

Primary antibody	Commercial source	Secondary antibody	Commercial source
PIF6: rabbit polyclonal antibody (anti-β5 integrin subunit).	Abcam	Biotinylated goat anti-rabbit IgG antibody	Vector labs
Q-20: rabbit polyclonal antibody (anti-αv integrin subunit).	Santa Cruz	Biotinylated goat anti-rabbit IgG antibody	Vector labs
LM609: mouse monoclonal antibody (anti-αvβ3 integrin)	Chemicon (Millipore)	Biotinylated horse anti-mouse IgG antibody	Vector labs
JBS5: mouse monoclonal antibody (anti- α5β1 integrin).	Chemicon (Millipore)	Biotinylated horse anti-mouse IgG antibody	Vector labs
C-11: mouse monoclonal antibody (anti-pan cytokeratin)	Abcam	Biotinylated horse anti-mouse IgG antibody	Vector labs

Table 7 Primary and secondary antibodies used in immunohistochemistry

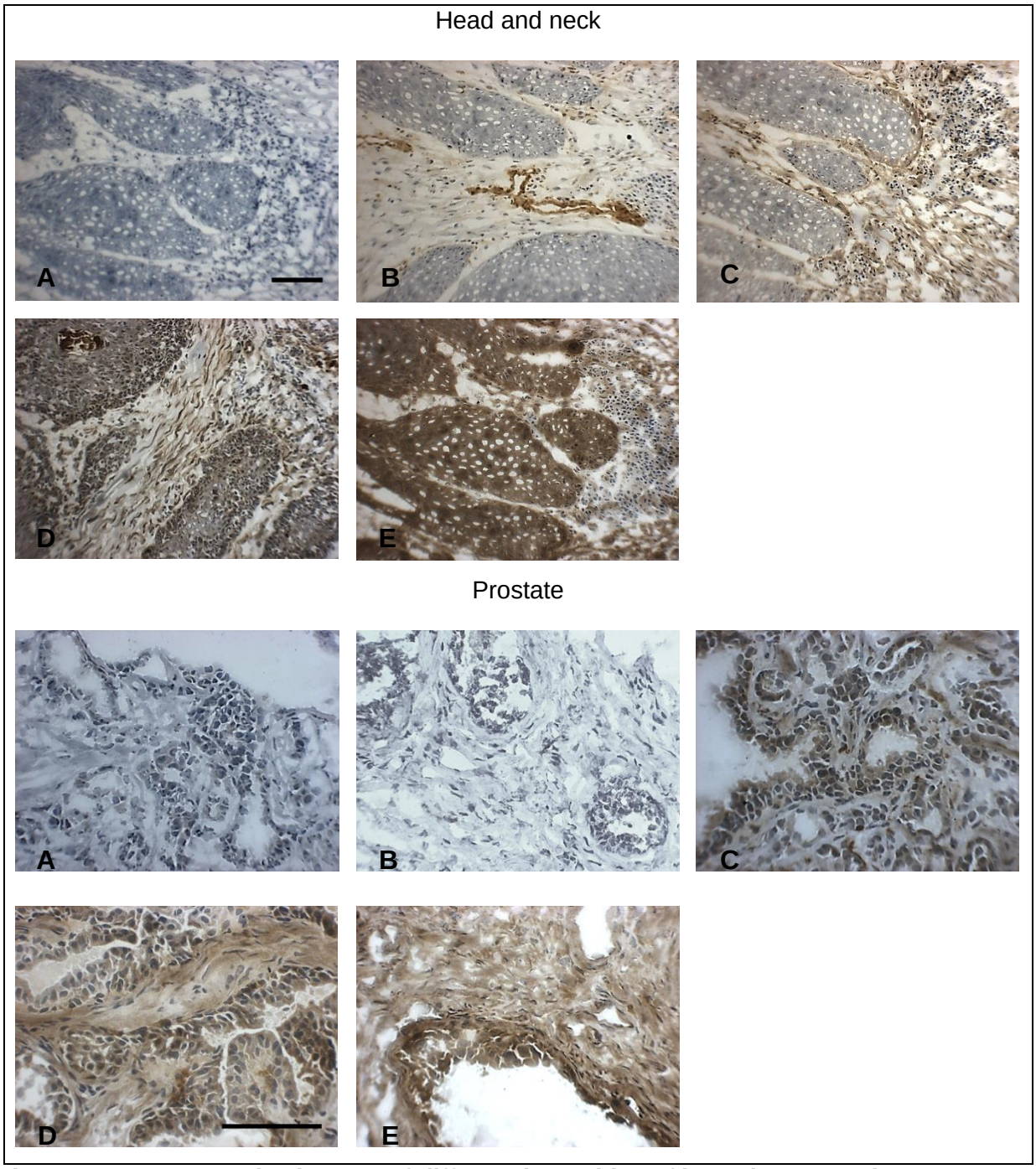


Figure 10 Representative images of different intensities of integrin expression
A: (0) no integrin expression; B: (1) weak expression; C: (2) moderate expression; D: (3) strong expression; E: (4) very strong expression. Scale bar = 100 µm.

2.4 RESULTS

2.4.1 The expression of integrins in fresh frozen human head and neck tissues

The distribution of αv, β5, αvβ3 and α5β1 integrins was assessed in normal epithelium, squamous cells carcinoma, endothelial cells and stroma Figure 12, Figure 13 and Figure 14 and Table 8.

The overall mean of the immunolabelling scores shows that αv, β5, αvβ3 and α5β1 integrin expression was higher in squamous cells carcinoma than in the control normal epithelium. Integrin αv was expressed in squamous cells carcinoma in almost all tumour samples (80% scored 4) and in the normal epithelium in normal tissues (80% scored 3). The difference between the expression of αv integrin subunits on normal and tumour samples was statistically significant (P = 0.01). Moreover, most of the normal epithelium in normal tissues lacked integrin αvβ3 (70% scored 0, and 30% scored 1). The increase in αvβ3 integrin expression from the normal epithelium in normal tissues to squamous cells carcinoma in tumour tissues (50% scored 2) was statistically significant (P = 0.02). A similar difference was observed for β5 and α5β1 integrins, with an increase in staining from the normal epithelium in normal tissues (50% scored 2, and 40% scored 0) and (60% scored 0) to squamous cells carcinoma in tumour tissue (40% scored 3 and 50 % scored 2) and (60% scored 3), respectively. The difference between the expression of β5 integrin subunits on normal and tumour samples was statistically significant (P = 0.05). The expression of α5β1 integrin was higher in squamous cells carcinoma than

the normal epithelium in control tissues; the difference in expression was statistically significant (P = 0.07) Table 9.

The expression of αvβ3, α5β1 and β5 integrins was very strong, moderate and weak respectively in both the tumour and control endothelial cells. The expression of these integrins was higher in tumour endothelial cells than normal endothelial cells except the expression of αv integrins was higher in the normal endothelial cells. The difference between the expression of αv and α5β1 in normal and tumour endothelial cells was statistically significant (P = 0.07 and 0.2).

The expression of αv, αvβ3, β5 and α5β1 integrins in tumour stroma was higher than in stroma of the control sample and the difference was statistically significant (P = 0.02, 0.08, 0.02 and 0.05 respectively).

The expression of αv in tumour stroma, αvβ3 and α5β1 in squamous cells carcinoma and α5β1 normal stroma in the group with lymphatic metastasis (N+) was significantly higher than in the group without metastasis (N0) (P = 0.13, 0.03, 0.13 and 0.13 respectively). The data did not show a statically significant trend but there was increase in metastasis correlated to the expression of αv in squamous cells carcinoma and αvβ3 and β5 in tumour stroma; this may reach significance in a larger study Table 9.

The expression of β5 in tumour endothelial cells and α5β1 in tumour stroma was correlated positively with the tumour size (P = 0.2 and 0.04 respectively). The data did not show a statically significant trend but there was increase in

tumour size correlated to the expression of αv in squamous cells carcinoma, αvβ3 in tumour endothelial cells and α5β1 in squamous cells carcinoma and tumour endothelial cells; this may reach significance in a larger study (see Table 10).

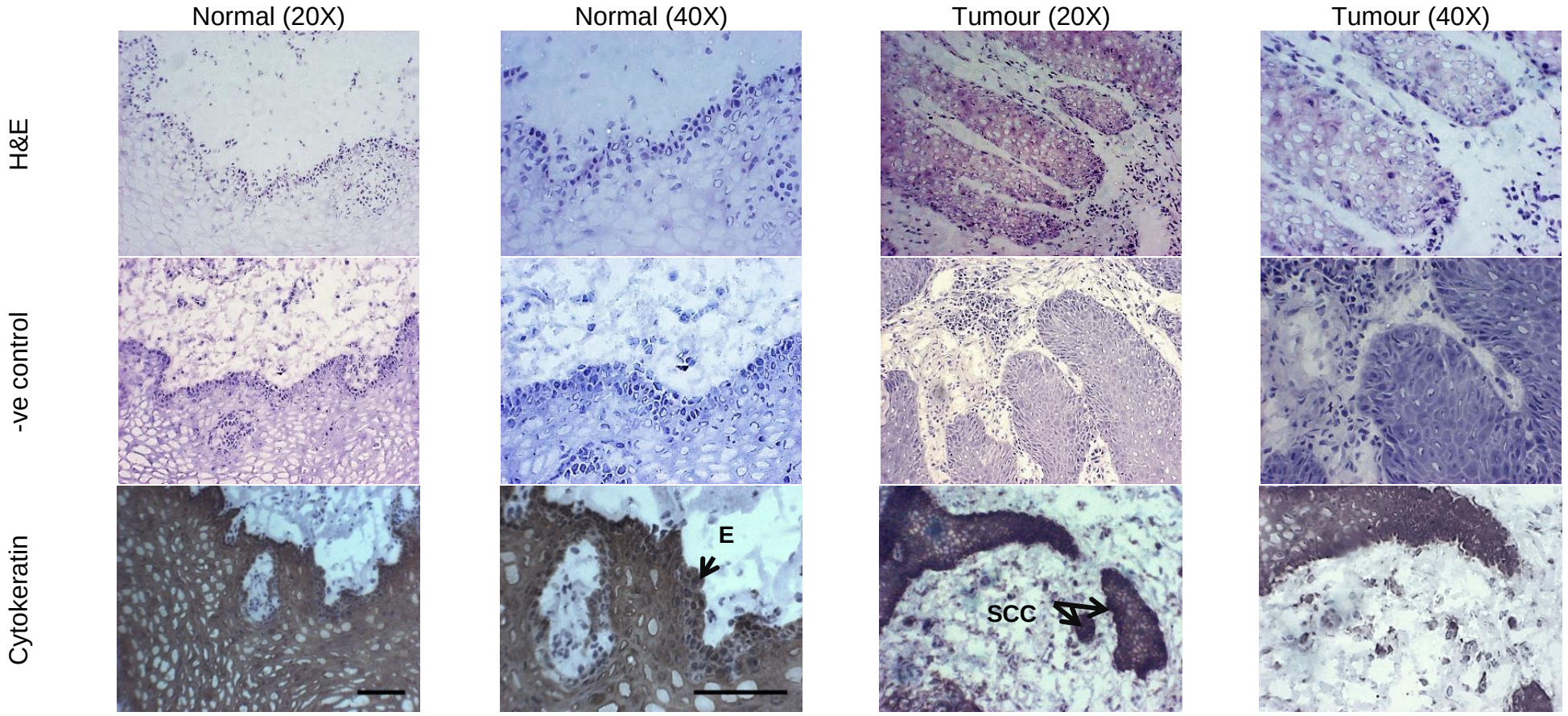


Figure 11 H & E Staining of fresh frozen head and neck tissues.
Representative images of H & E staining of normal and tumour tissue (First row). Negative control without primary antibody (second row). Immunolabeling with C-11 antibody showed strong cytoplasmic expression of cytokeratin in normal and tumour tissues. E, epithelium; SCC, squamous cell carcinoma. Bar length = 100 µm.

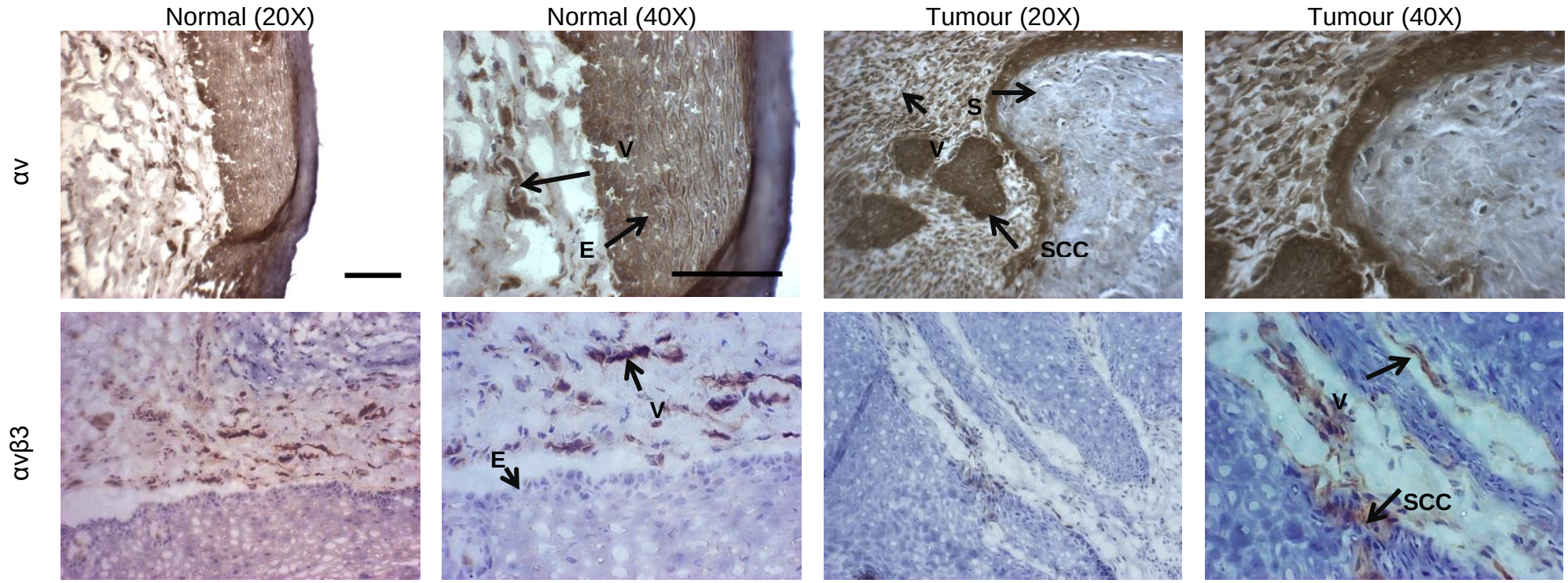


Figure 12 Representative images of immunohistochemical expression patterns of αv and αvβ3 integrins in head and neck samples.
Q-20 and LM609 antibodies were used to detect αv and αvβ3 integrins in fresh frozen tissues. E, epithelium; SCC, squamous cell carcinoma; V, blood vessels; S, stroma. Bar length = 100 μm

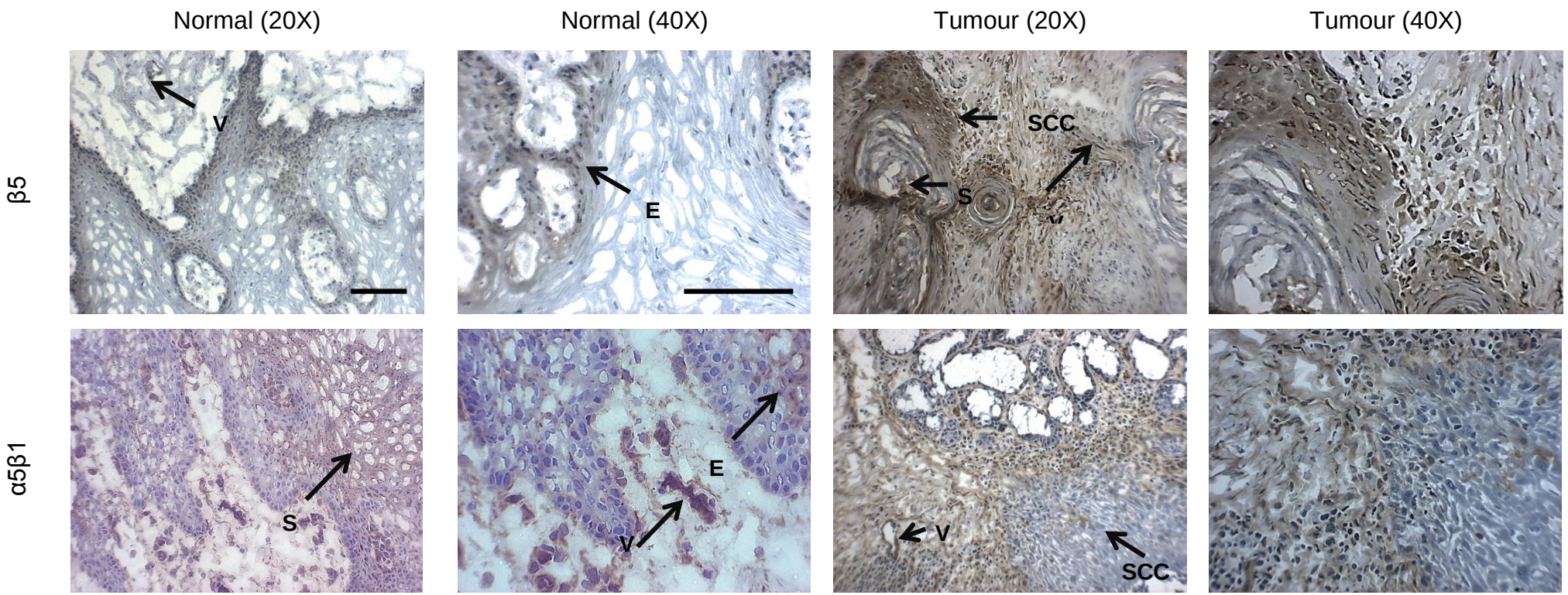


Figure 13 Representative images of immunohistochemical expression patterns of β5 and α5β1 integrins in head and neck samples
PIF6 and JBS5 antibodies were used to detect β5 and α5β1 integrins in fresh frozen tissues. E, epithelium; SCC, squamous cell carcinoma; V, blood vessels; S, stroma. Bar length = 100 μm.

	Squamous cells carcinoma	Normal epithelium	Endothelial cells	Stroma
αv				
Normal	0	2.6 ± 0.9	2.6 ± 0.9	0.4 ± 0.8
Tumour	3.7 ± 0.64	0	1.4 ± 1.4	1.8 ± 1.5
αvβ3				
Normal	0	0.3 ± 0.5	3.7 ± 0.5	0.2 ± 0.6
Tumour	1.1 ± 0.9	0	3.8 ± 0.6	0.8 ± 1
β5				
Normal	0	1.3 ± 1	1.5 ± 1	0.2 ± 0.6
Tumour	2.2 ± 0.9	0	1.3 ± 1	1.2 ± 1
α5β1				
Normal	0	0.9 ± 1	1.8 ± 1	1.5 ± 1
Tumour	2.1 ± 1	0	2.2 ± 1	2.4 ± 0.9

Table 8 Integrin expression in head and neck tissues.

20 samples from head and neck were used: 10 tumour tissues and 10 non-cancerous tissues originating from the same patient. Results are shown as the means ± SD for integrin expression levels on the specified tissue.

A		B	
IHC in the squamous cells carcinoma vs normal epithelium in control	P value	HIS in the tumour cells in group with (N+) vs (N0)	p value
αv	0.01	αv	0.5
αvβ3	0.02	αvβ3	0.03
β5	0.05	β5	0.4
α5β1	0.07	α5β1	0.1
HIS in the tumour endothelial cells vs endothelial cells in control	P value	HIS in tumour endothelial cells in group with (N+) vs (N0)	P value
αv	0.07	αv	0.7
αvβ3	0.6	αvβ3	0.7
β5	0.7	β5	1
α5β1	0.2	α5β1	1
HIS in the tumour stroma vs stroma in control	P value	HIS in the tumour stroma in group with (N+) vs (N0)	P value
αv	0.02	αv	0.1
αvβ3	0.08	αvβ3	0.7
β5	0.02	β5	0.3
α5β1	0.05	α5β1	1

Table 9 Statistical analysis of the immunohistochemistry scores of integrin expression in HNSCC.

Comparison of integrin expression (A) between normal tissues and tumour tissues in paired samples from the same patient, and (B) patients with lymph node metastasis (N+) versus no lymph node metastasis (N0).

Integrin expression in squamous cell carcinoma and correlation with tumour size	P value
αv	0.8
αvβ3	0.8
β5	1
α5β1	0.7
Integrin expression in tumour endothelial cells and correlation with tumour size	P value
αv	0.7
αvβ3	1
β5	0.2
α5β1	0.7
Integrin expression in tumour stoma and correlation with tumour size	P value
αv	0.9
αvβ3	1
β5	0.3
α5β1	0.04

Table 10 Statistical analysis for correlation between the immunohistochemistry score of integrin expression in tumour tissues with tumour size (T)

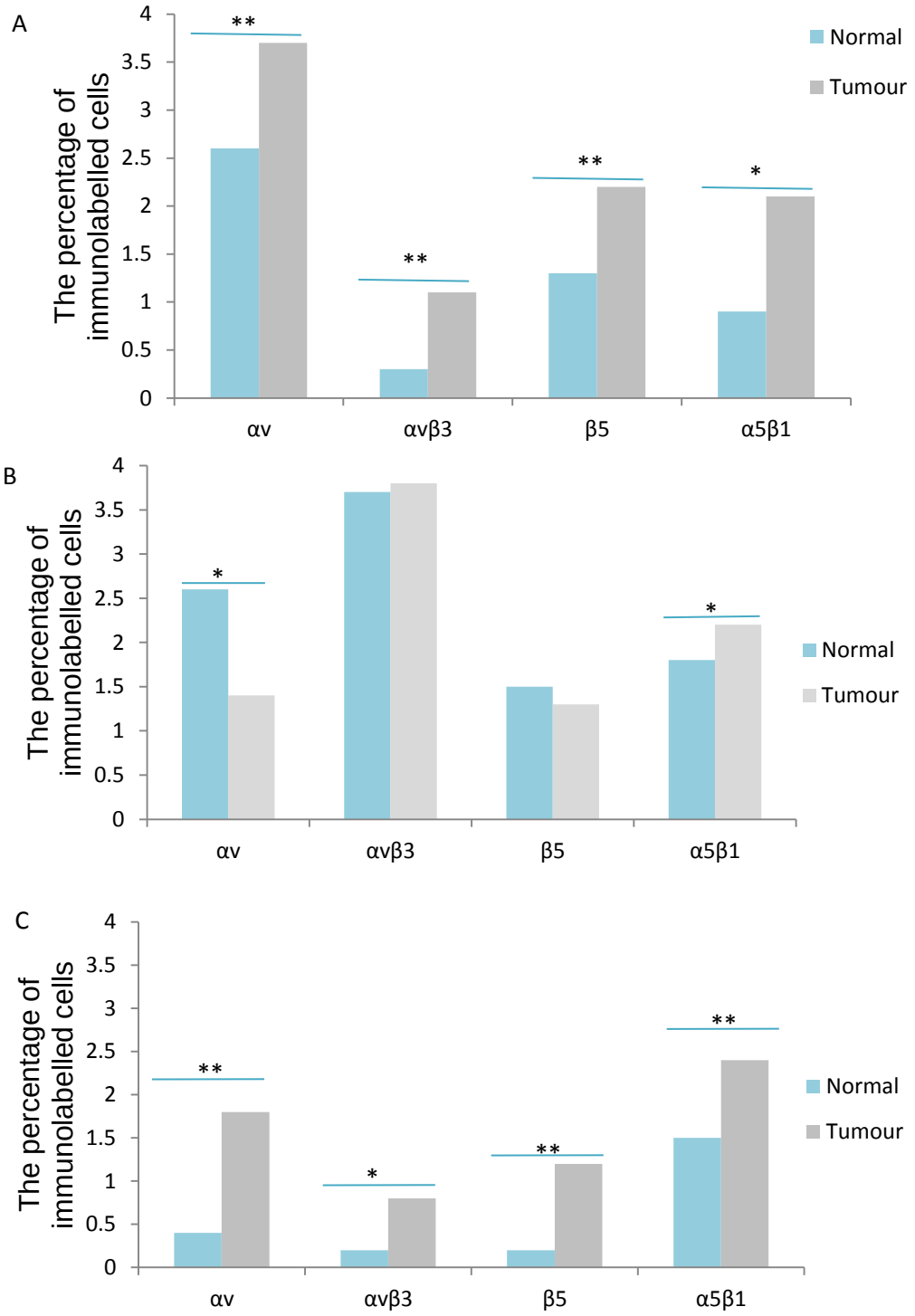


Figure 14 Integrin expression (% immunolabelled cells) in normal and tumour head and neck tissues.

Ten head and neck samples were used, comprising 10 non-cancerous tissues and 10 tumour tissues originating from the same patient. The percentage of immunolabelled cells in A, normal epithelium and squamous cells carcinoma; B, endothelial cells; C, stroma, are shown as the means ± SD. The asterisk * indicates $P < 0.2$, ** indicates $P < 0.05$.

2.4.2 The expression of integrins in prostate tissues

αvβ3 and α5β1 integrins were often expressed on the cell membrane, whereas αv and β5 integrin subunits were cytoplasmic (Figure 15 and Figure 17). The αv integrin subunit was strongly expressed in tumour cells (66% score 3), moderately in stroma (77.7% score 2) and very weakly expressed in endothelial cells. Integrin αvβ3 was rarely expressed in tumour cells and endothelial cells (44% and 77% score 0, respectively) and highly expressed in stromal cells (100% score 3). The β5 integrin subunit expression was high in tumour cells (22% score 4, 33% score 3 and 44% score 2) and very low in stroma and endothelial cells (77% and 44% score 0, respectively). Integrin α5β1 was strongly expressed in stromal cells (100% score 3) and moderate in tumour cells (66% score 2) but was not expressed in endothelial cells (100% score 0) (Figure 15, Figure 16 and Figure 17). Expression levels of αv, β5 and α5β1 integrins in the tumour cells were significantly higher than in normal cells ($P = 0.008$, $P = 0.0102$ and $P = 0.0102$) respectively. These data did not show any correlation between integrin expression and Gleason scores (Table 11).

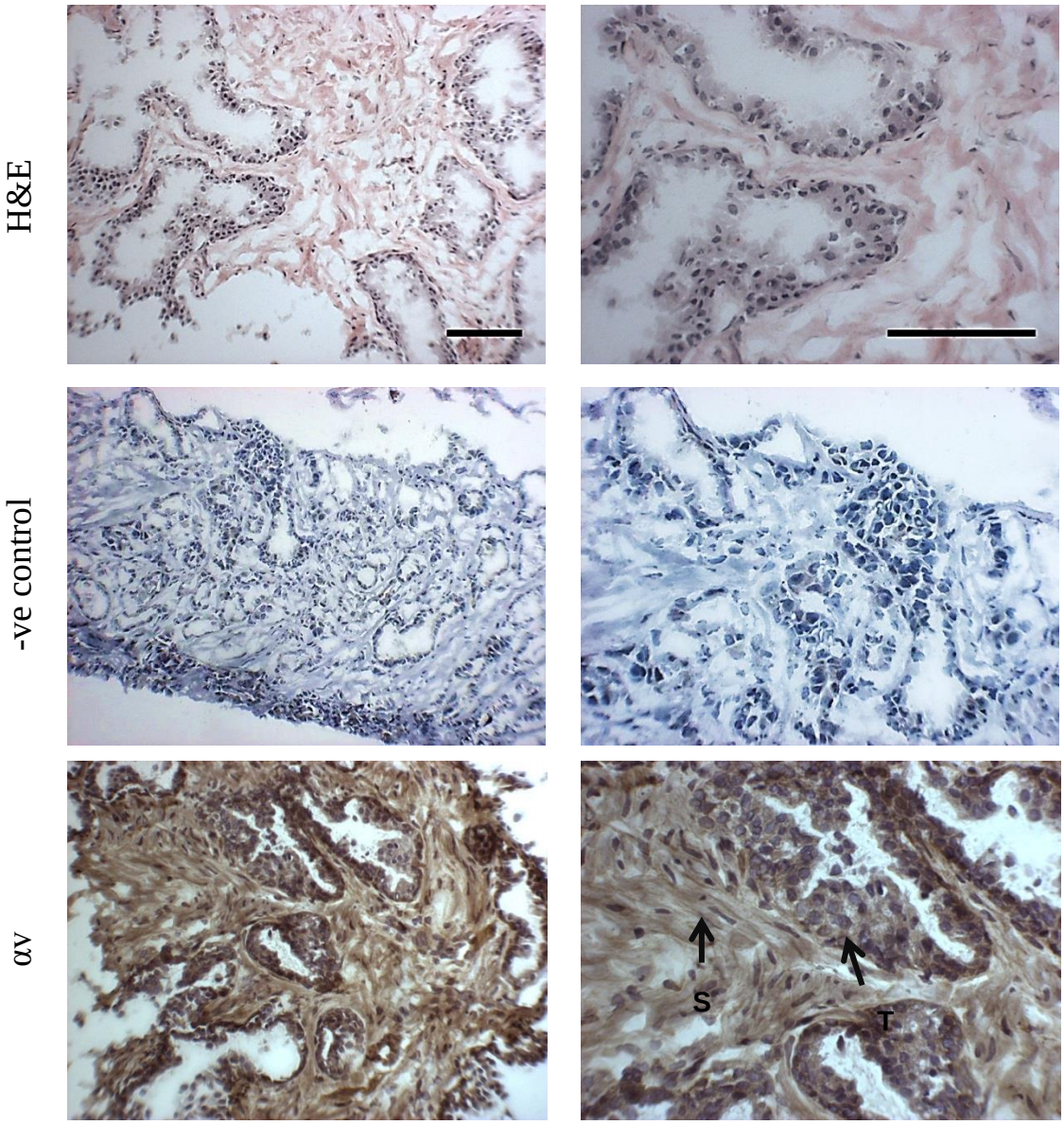


Figure 15 Staining of fresh frozen prostate tissues.
First row: Representative images of H & E staining where nucleus stains blue and the cytoplasm red. Second row: Negative control without primary antibody. Third row: Immunolabelling with Q-2 antibody shows cytoplasmic expression of αv in tumour cells and stroma. T, tumour; S, stroma. Bar length = 100 μm.

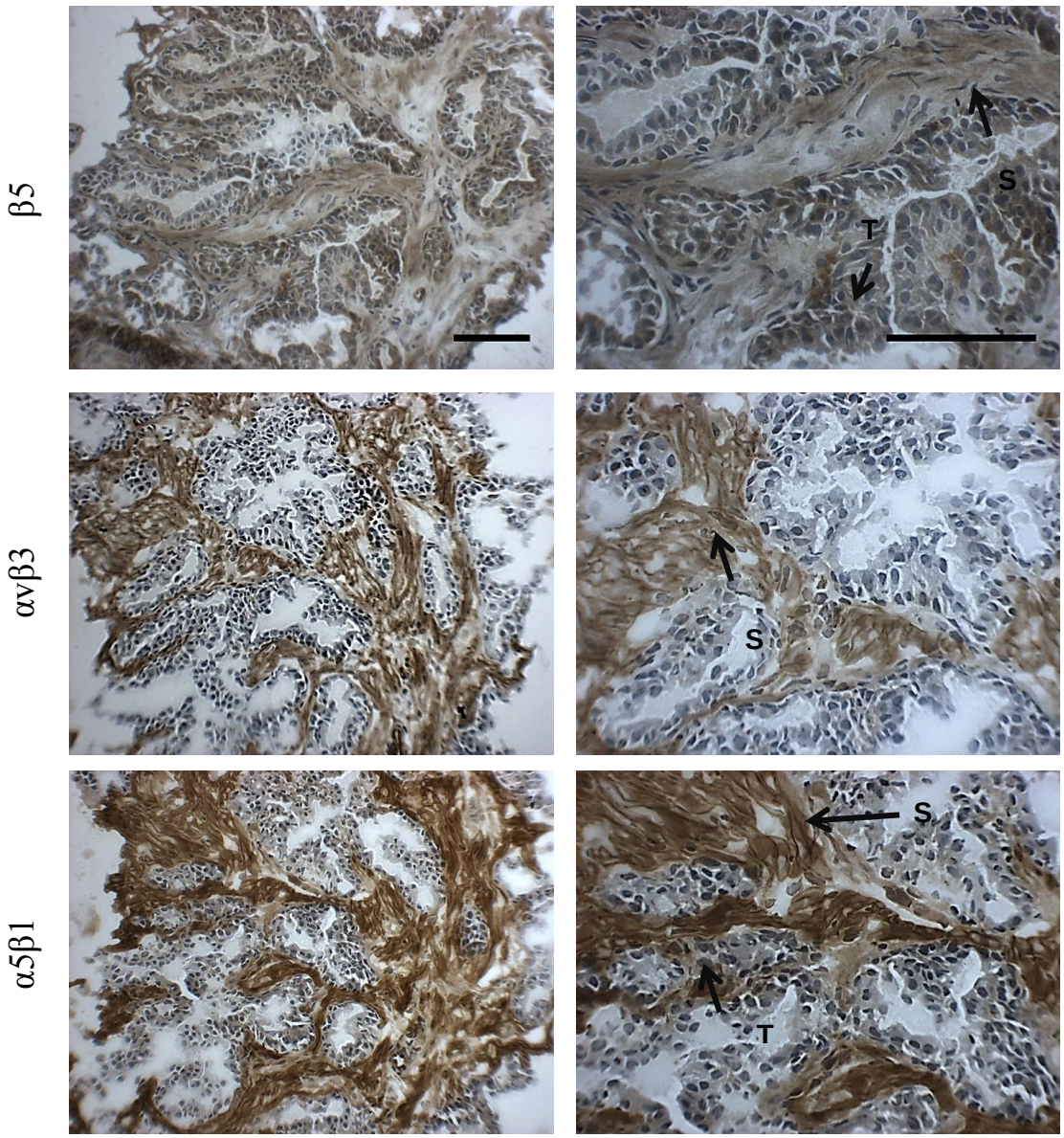


Figure 16 Representative images of immunohistochemical expression patterns of αvβ5, αvβ3 and α5β1 integrins in head and neck samples

αvβ3 (LM906) and α5β1 (JBS5) antibodies gave membranous staining whereas αvβ5 (PIF6) antibody gave cytoplasmic staining. T, tumour; S, stroma. Bar length = 100 μm.

	Expression on tumour cells	Expression on normal cells	IHC in the tumour cells vs normal cells (P value)	Expression on endothelium	Expression on stroma
αv	2.75 ± 0.5	0 ± 0	0.008	0.3 ± 1	2.125 ± 0.6
αvβ3	0.85 ± 1	0 ± 0	0.08	0.7 ± 1.3	3 ± 0.9
β5	3 ± 0.8	0.3 ± 1	0.01	0.5 ± 1.1	1 ± 0.9
α5β1	2 ± 0.8	0 ± 0	0.01	0 ± 0	3 ± 1.2

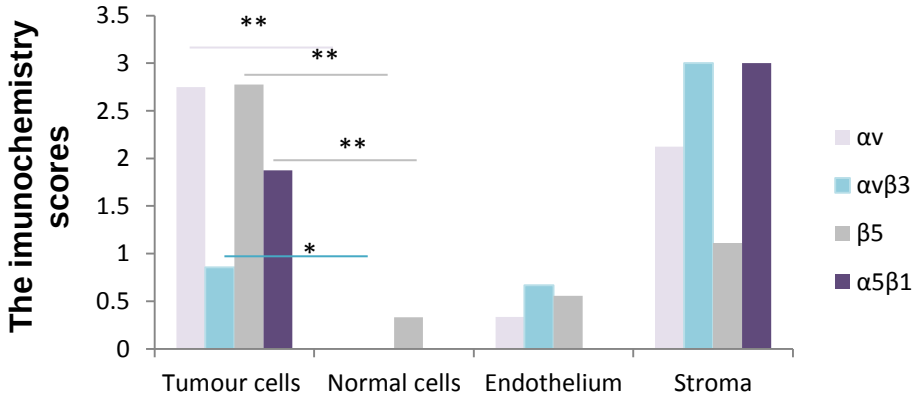


Figure 17 Integrin expression in prostate tissues
Eight samples from the prostate were used. Results are shown as the means ± SD for integrin expression levels in the specified tissue. The asterisk * indicates P < 0.4, ** indicates P< 0.05.

IHC in the tumour cells in group with Gleason score 6 and 7	p value
αv	0.2
αvβ3	0.7
β5	1
α5β1	0.5
IHC in the endothelial cells in group Gleason score 6 and 7	P value
αv	0.6
αvβ3	n/a
β5	0.6
α5β1	n/a
IHC in stromal cells in group with Gleason score 6 and 7	P value
αv	n/a
αvβ3	n/a
β5	0.37
α5β1	n/a

Table 11 Statistical difference between the immunohistochemistry score of integrin expression with Gleason score 6 vs 7.

2.5 Discussion

In this study, the expression of αv, β5, αvβ3 and α5β1 integrins in head and neck fresh frozen tissues, comprising 10 tumour tissues and 10 normal tissues originating from the same patient, and in fresh frozen prostate tissues from nine patients, were evaluated by immunohistochemistry. This allowed analysis of the distribution of these integrins in tumour cells, squamous epithelial cells, endothelial cells and stroma.

Previous studies of integrin distribution in tissue have generally used fresh frozen tissues (Max et al., 1997, Fabricius et al., 2011, Denadai et al., 2013) because antibodies that react with FFPE tumour tissue were not available. Recently, a set of antibodies, which allow the labelling of integrin complexes in FFPE tissue have been produced and validated at the molecular, cellular and tissue levels but are not yet available for research use (Goodman et al., 2012). Inability to obtain these antibodies for this study limits the sample number that can be analysed because retrospective analysis of FFPE sections that are accessible from stored samples is not possible with the antibodies that label integrin complexes in fresh frozen tissues.

2.5.1 Head and neck tissues

This study demonstrated frequent expression of β5, αvβ3 and α5β1 integrins in tumour cells; importantly, normal squamous epithelium cells weakly express β5, αvβ3 and α5β1 integrins. The difference between the expression of αv, β5, αvβ3 and α5β1 on normal and tumour samples was statistically significant

suggesting up-regulation of these integrins in tumourigenesis. Strong αvβ5 integrin expression in tumour cells was reported for tongue (Kurokawa et al., 2008), oral squamous cell carcinoma (Jones et al., 1997) and laryngeal squamous cell carcinoma, and its expression is associated with lymphatic metastasis and angiogenesis (Li et al., 2013). In addition, strong expression of αvβ5 integrins in tumour cells was also reported in other cancers, such as colorectal cancer and melanomas (Goodman et al., 2012). Fabricius *et al*., previously found that the expression of αvβ5 and α5β1 integrins in squamous cell carcinoma in oral cancer tissues was significantly higher than that of squamous epithelium in normal tissues (Fabricius et al., 2011). The expression of α5β1 integrins in head and neck tissues was associated with invasion and metastasis, and with a poor prognosis (Shinohara et al., 1999, Vitolo et al., 2000, Charalabopoulos et al., 2005).

I found that although αvβ3 integrin was weakly expressed in tumour cells, the expression was significantly higher than in noncancerous squamous epithelium ($P = 0.02$). These results are consistent with studies in malignant glioma and melanoma, which indicated that tumour cells express higher levels of αvβ3 than normal tissues (Albelda et al., 1990). In contrast, Fabricius *et al*. reported a low expression of αvβ3 integrin in head and neck tumour tissues (Fabricius et al., 2011) and failure of a clinical trial of Cilengitide was suggested to be due to lack of expression of the target integrins in this tumour type (Vermorken et al., 2014). Our results showed that αvβ3, α5β1 and β5 expression in tumour cells and β5 in tumour stroma was significantly higher in the group with lymph node metastasis (N+) than in tumour cells and stroma in the group without lymph

node metastasis (N0) (P = 0.03, 0.4, 0.1 and 0.3 respectively). This finding supports preclinical studies that have demonstrated the implications of αvβ5 integrin in metastasis of head and neck cancer (Li et al., 2011, Li et al., 2013, Lu et al., 2009, Koivisto et al., 2000).

Integrins αvβ3, αvβ5 and α5β1 are implicated in vascular development and the blocking of these integrins inhibits angiogenesis and tumour growth (Kim et al., 2002, Brooks et al., 1995, Reardon et al., 2008, Kumar et al., 2001). The present work identifies that the expression of αvβ3 and α5β1 integrins was slightly higher in endothelial cells within tumour than normal tissues and difference in the expression of α5β1 was statistically significant (P = 0.2 in this small study). This finding is in agreement with Fabricius *et al*. who showed high αvβ3 and α5β1 integrin expression in tumour endothelia cells compared to endothelial cells in control normal tissues (Fabricius et al., 2011). Similarly a study by Max *et al*. reported strong αvβ3 integrin expression in tumour blood vessels in fresh frozen human tissue from different epithelial tumours (Max et al., 1997). The expression of αvβ3 and α5β1 integrins indicates their role in angiogenesis as other studies showed the involvement of these integrins in endothelial cell proliferation, differentiation and migration (Eliceiri et al., 1998) (Lee and Ruoslahti, 2005) (Kim et al., 2002).

Surprisingly, I found the expression of the αv and β5 integrin subunits to be low in the endothelial cells of tumour tissues. This contrasts with the results of Li et al. (2013), who found that the αvβ5 integrin is upregulated in the tumour blood vessels.(Li et al., 2013).

In the present study, α5β1 integrin was expressed more frequently than αv, β5 and αvβ3 integrins in the normal stroma of the control samples. However, in tumour stroma, an increased expression of αv, β5, αvβ3 and α5β1 integrins was observed, and the expression of these integrins in tumour stroma was significantly higher than in the normal stroma. Fabricius *et al*. reported also that the expression of αvβ3, αvβ5 and α5β1 integrins was higher in tumour stroma than in normal stroma in control tissue, and found that αvβ5 integrins were markedly expressed in tumour stroma rather than in normal stroma in control tissues (Fabricius et al., 2011). The present study suggests head and neck cancer would be a suitable clinical problem to address by multi-integrin targeting.

It is important to note that the small number of samples in this pilot study seriously limits the extent of statistical analysis. As discussed, a larger sample size would be required to show statistically significant observable trends. Analysis of a larger dataset may reveal further associations between integrin expression, tumour size, and metastasis. Scoring integrin expression on a continuous scale would also be more useful for statistical analysis.

2.5.2 Prostate tissues

This study showed that in prostate tissues, αvβ3 integrin was prevalent in stroma, and occasionally on tumour cells and endothelial cells, and was not detected in squamous epithelium cells. Zheng *et al*., previously observed a similar expression profile for αvβ3 integrin in prostate cancer cells but not in

normal prostate epithelial cells (Zheng et al., 1999) whereas Hess *et al*. (2014) and Goodman *et al*. (2012) reported the expression of αvβ3 integrins on blood vessels only. Differences between the results could be due to experimental procedures, the patient demographic investigated, type of prostate tumour and invasiveness of the tumour.

These data showed that αv and β5 integrin subunits was extensively expressed in tumour cells, occasionally in stroma and rarely in endothelial cells. These results are in agreement with those of Goodman *et al*., who found that prostate cancer samples expressed essentially only αvβ5 integrins in tumour cells and stroma (Goodman et al., 2012). Hess *et al*. reported a high expression of αvβ5 integrin in prostate cancer cells which was correlated significantly with the Gleason pattern (Hess et al., 2014). I found that β5 integrin expression in tumour stroma was significantly higher in the group with a Gleason score of 7 than in the group with a score of 6.

In the present study, α5β1 integrin was strongly expressed in stroma and moderately in tumour cells, but it was not detected in endothelial cells or squamous epithelial cells. This is in contrast to observations noted by Chen *et al*. (2006) and Nagle *et al*. (1994), who found that squamous epithelium cells expressed higher levels of α5β1 integrins than tumour cells (Chen et al., 2006, Nagle et al., 1994).

Again, this analysis was limited by the very small number of patient samples available. Some samples did not allow for the scoring of integrin expression on

all tissue types, and some patient data was incomplete. As a result, it was difficult to observe any trends between integrin expression and tumour progression.

2.6 Conclusion

Despite the very small number of samples available for use in this pilot study, αv, β5, αvβ3 and α5β1 have been identified as significantly overexpressed in tumour tissues, and there are trends suggesting a correlation with metastasis and tumour size in head and neck cancer. Further analysis of a larger number of samples, preferably scoring by percentage integrin expression, will be required to unambiguously state that αvβ5, αvβ3 and α5β1 are a promising target for cancer therapy. In order to further investigate the effect of integrin expression on tumour cell function, and to provide models for use in drug development, it will be necessary to identify cell line models which match the integrin expression of clinical tissues.

3 CHAPTER 3: Characterisation of integrin expression in *in vitro* and *in vivo* cell line models

3.1 Introduction

Cell-cell adhesion and interaction of cell integrins with extracellular matrix proteins play crucial roles in each step of tumour progression: integrins are implicated in tumour cell proliferation, migration, survival and angiogenesis.

For this reason, the distribution and expression of integrins has been extensively studied in clinical samples, and in *in vitro* and *in vivo* models (Goodman et al., 2012, Terry et al., 2014, Seguin et al., 2012, Stachurska et al., 2012, Roman et al., 2010). The expression and role of integrins, specifically the RGD-binding integrin subfamily in cancer progression, are described in Chapter 1. In this chapter, the expression of α5, αv, β5 and β3 integrin subunits was evaluated *in vitro* and *in vivo* to select appropriate *in vitro* and *in vivo* models to evaluate the efficacy of novel integrin antagonists as drug targets.

3.2 Aim of this study

Interaction of cell integrins with ECM proteins is associated with cell migration, invasion and tumour metastases. Moreover, cancer cell survival depends on an ability to bind with the ECM proteins. Therefore, this study investigated the involvement of αvβ5, αvβ3 and α5β1 integrins in cancer progression. In particular:

- the expression of αv, β3, β5 and α5 integrin subunits in a panel of tumour cell lines: tongue squamous carcinoma cells OSC-19; prostate cancer cells PC-3 and DU-145; human breast carcinoma cells MCF-7; MCF-7ADR/NCRI ADR-RES cells as a drug-resistant model; colorectal adenocarcinoma cells DLD-1 and HT-29; melanoma cells M14 and human umbilical vein endothelial cells HUVEC using immunofluorescence and Western blot in order to select cell line models with high or low expression of αv, β5 and α5 integrin subunits to use in functional studies.

- The expression of αv, β3, β5 and α5 integrin subunits in a panel of FFPE and fresh frozen human xenograft tumours grown in mice using immunohistochemistry and Western blot. Expression of these integrin subunits in fresh frozen xenograft tissues was compared with *in vitro* 2D cell cultures to investigate the effect of the tumour microenvironment on the expression of integrins. Confirmation of the expression profile *in vivo* will allow the selection of models to be used once novel antagonists eventually progress to *in vivo* screening.

3.3 MATERIALS AND METHODS

3.3.1 Reagents

All cell culture materials were purchased from Sigma-Aldrich unless indicated. For immunofluorescence and Western blot, reagents were purchased from several sources, as described in the relevant section below.

3.3.2 Cell lines and Cell culture

A panel of cell lines reported in the literature to have high or low expression of αv, β5 and α5 integrin subunits were selected: human breast carcinoma cells MCF-7; MCF-7ADR/NCRI ADR-RES cells as a drug-resistant model (Taherian et al., 2011, Nista et al., 1997); prostate cancer cells PC-3 and DU-145 (Stachurska et al., 2012, Witkowski et al., 1993); colorectal adenocarcinoma cells DLD-1 and HT-29 (Haier et al., 1999, Petersen et al., 2013); tongue squamous carcinoma cells OSC-19 (Kawahara et al., 1995); melanoma cells M14 and human umbilical vein endothelial cells HUVEC (Goodman et al., 2012). All cell lines were purchased from NCI, except for DU-145 and DLD-1 which were obtained from ATCC. Cells were regularly grown in 75 cm^2 cell culture flasks (T-75) flasks (Corning) at 37 °C, 5% CO_2 and 95% air as monolayer cultures in RPMI-1640 culture medium containing 10% foetal bovine serum (FBS), sodium pyruvate (1 mM) and L-glutamine (2 mM).

When the cells had become 70-80% confluent, they were passaged by trypsinisation. The old medium was aspirated using a pipette, cells were

washed 2 times with 10-15 ml of HBSS prior to the addition of 3 ml of trypsin-EDTA and the mixture was incubated for 5-15 minutes at 37 °C until the cells were completely detached and separated into single cells. 10 ml of complete media was added and the cell suspension was centrifuged at room temperature for 5 minutes at a speed of 1000 RPM. The supernatant was discarded and the cell pellets were re-suspended in 10 ml of fresh RPMI medium. The required amount of cell suspension was aliquoted into a sterile non-vented T-75 flask. Cells were discarded after 10 passages and replaced with fresh stocks because cell lines at high passage numbers experience alterations in cell morphology, response to stimuli, growth rates, and protein expression, compared to lower passage cells.

To determine the cell density in a cell suspension, a Neubauer's modified haemocytometer was used. A cell suspension (10 µl) was transferred with a pipette to the edge of both chambers of the haemocytometer. Due to capillary action, the mixture was drawn underneath the cover slip. The cell count was performed in 10 chambers (8 corner squares and 2 middle squares) by using a low-power (x10) objective microscope. The number of cells per ml was calculated as the following:

Number of cells per ml = average number of cells counted × dilution factor $\times 10^4$

3.3.3 *In vivo* xenograft modelling

Animal procedures were performed according to a protocol approved by the UK Home Office and in accordance with the UK National Cancer Research Institute Guidelines for the Welfare of Animals (Workman et al. 2010). Immunodeficient

Balb/C Nu/Nu mice aged 6 to 8 weeks obtained from Harlan (Loughborough, UK) were injected subcutaneously with human tumour cells and when the tumour reached 500 mm^3 in volume, the mice were sacrificed and the tumour extracted. Some tumours were snap-frozen and stored in liquid nitrogen and some were embedded in paraffin. Four cell lines were used in this study: OSC-19, PC-3, HT-29 and MCF-7. *In vivo* xenograft modelling was done by Patricia Cooper.

3.3.4 Immunofluorescence

3.3.4.1 General protocol

A cell suspension was prepared and 3×10^5 cells/ml seeded on cover slips in 6 well plates because this concentration was sufficient to give 70% confluency after overnight incubation at 37 °C. The culture medium was aspirated from each well. Cells were washed twice with 1 ml of HBSS at room temperature. The cells were then fixed by adding 1 ml of one of the fixative agents: acetone (1 second at room temperature), methanol (15 minutes at −20 °C) or 4% paraformaldehyde (15 minutes at room temperature) and the plate were left to dry. The plate was left in –20 °C overnight. The cells were rehydrated in PBS (Sigma-Aldrich) and blocked by adding 300 µl of one of the blocking agents: BSA (Sigma-Aldrich), goat serum or donkey serum (Millipore) for 1 hour at room temperature (approx. 22 °C). The blocking was removed and 100 µl of diluted primary antibody was added and incubated at overnight at 4 °C or 1 hour at room temperature or one hour at 37 °C. Primary antibody was withheld from

negative controls. The cells were washed three times for 10 minutes with blocking agent and then incubated with 100 µl of diluted secondary antibody for 1 hour in dark at room temperature. Cells were washed three times for 10 minutes with PBS. The cover slips were mounted with Mounting Medium with DAPI (Vector Laboratories) and left in the freezer in the dark until dry. The primary and secondary antibodies which were used are summarized in Table 12. Also the steps which were followed to optimize the antibodies are shown in Figure 18.

Antibody	Company	Secondary antibody	Company
[ab15459]: Rabbit polyclonal anti- β5.	Abcam	Alexa Fluor 546 donkey anti rabbit IgG	Millipore
[Q-20]: Rabbit polyclonal anti-αv.	Santa Cruz	Alexa Fluor 546 donkey anti rabbit IgG	Millipore
[C-9]: Mouse monoclonal anti-α5.	Santa Cruz	Alexa Fluor 488 F(ab)2 fragment of goat anti mouse	Millipore
(B-7): Mouse monoclonal anti-β3.	Santa Cruz	Polyclonal rabbit anti-mouse immunoglobulins conjugated with tetramethylrhodamine isothiocyanate (TRITC)	Dako

Table 12 Primary and secondary antibodies used in immunofluorescence studies for detection of αv, β5, α5 and β3 integin subunits.

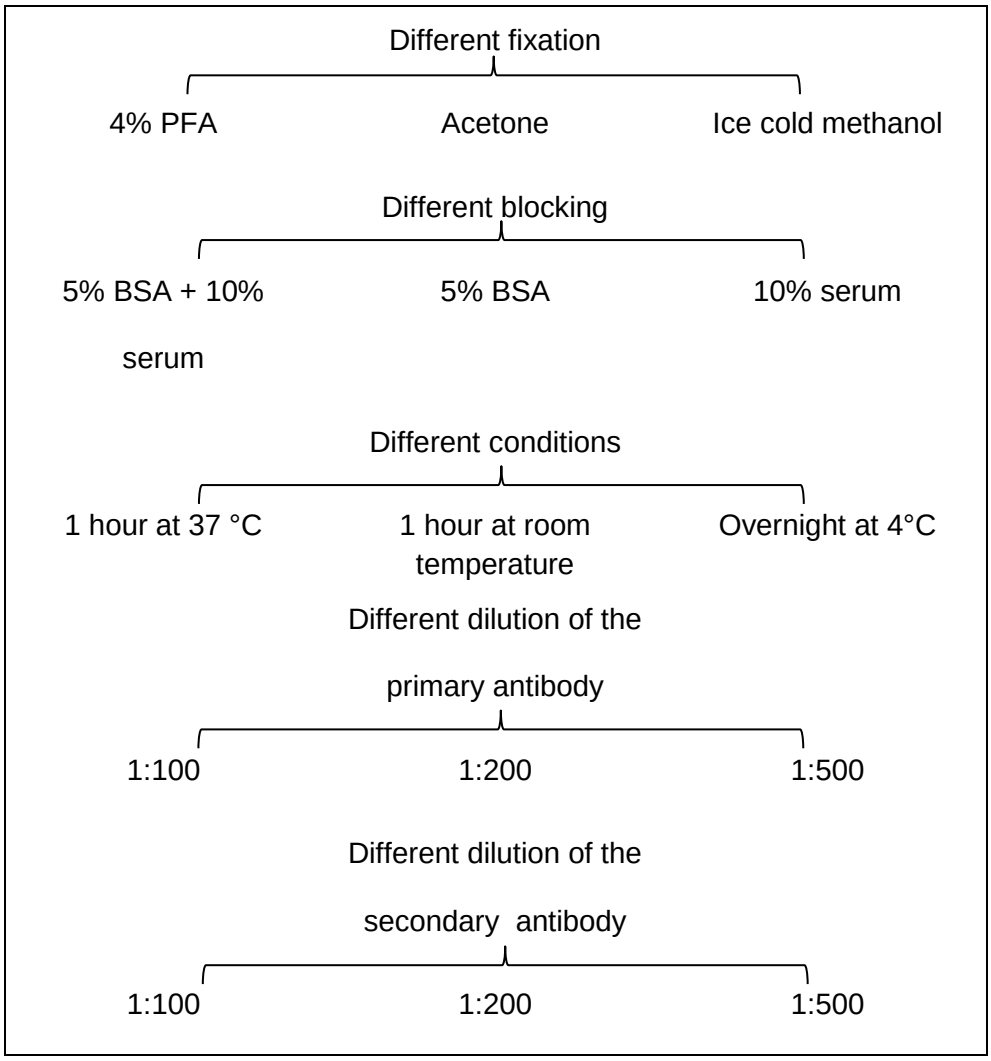


Figure 18 The steps which were followed to optimise the immunofluorescence staining technique

PFA; Paraformaldehyde, BSA; Bovine serum albumin.

3.3.5 Western blot

3.3.5.1 Protein extraction from cells

Three 75% confluent T-75 flasks were used to collect the cells from each cell line. Each flask was washed once with 10 ml of HBSS. After washing, a further 5 ml of HBSS was added and the cells were gently scraped by cell scraper and collected in an Eppendorf tube. The cell suspension was centrifuged at a speed of 13,000 rcf for 5 minutes at 22 ºC. The liquid phase was discarded and the pellet was resuspended in 2 X pellet volume of protein extraction buffer. Two lysis buffers were used to extract the protein: urea lysis buffer [71.5 µl of PIC with EDTA (Roche), 7 M urea, 2 M thiourea, 4% CHAPS, 0.1 % SDS and 0.05% sodium deoxycholate (Sigma-Aldrich) in PBS] and Triton lysis buffer [100 mM tris buffer, 300 mM NaCl, 5 mM EDTA and 2% Triton (Sigma-Aldrich)].

3.3.5.2 Protein extraction from xenograft tissues:

Human xenograft frozen tissue samples were placed on ice to prevent degradation by proteases and cut by a clean blade into approximately 2 mm^3 pieces. One piece was placed in Eppendorf tubes with 100 µl of RIPA lysis buffer [10 µl 10% SDS, 25 µl 10% sodium deoxycholate (Sigma-Aldrich), 71.5 µl of PIC with EDTA (Roche) in 893.5 µl PBS] for 30 minutes at room temperature with constant vortexing. The samples were sonicated 9 times for 3 cycles of 3 seconds with cooling on ice between each time to avoid overheating. The homogenate was centrifuged at a speed of 13,000 rcf for 20 minutes at 4 ºC.

The liquid phase was extracted to a new tube whereas the pellet was incubated with 100 µl of urea lysis buffer [71.5 µl of PIC with EDTA (Roche), 7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT (Sigma-Aldrich) in PBS] for 30 minutes at room temperature with constant vortexing. The mixture was then treated with RIPA lysis buffer as described above until the tissue was totally homogenised. The proteins were aliquoted and stored at −80 °C for later use.

3.3.5.3 Determination of protein concentration

The Bradford assay was used to determine the concentration of the protein in each sample by comparison to a standard curve. 1 mg of BSA was dissolved in 1 ml of water. 6 series of protein standards (1 ml each) were prepared as 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml BSA and blank by serial dilution. 45 µl of water and 5 µl of sample were added to the sample tube and mixed by vortex. 1.5 ml of Bradford reagent (Sigma-Aldrich) was mixed with 50 µl of standards, blank, and sample and incubated at room temperature for 15 minutes. The optical density of each standard and unknown sample was measured by spectrophotometer (Cary 50 BIO UV-Visible) at 595 nm. The protein concentration for the test sample was obtained from the standard curve.

3.3.5.4 SDS-Polyacrylamide Gel Electrophoresis

Equivalent amounts of protein determined by the Bradford assay were mixed with 1 volume of sample buffer [3.55 ml of water, 1.25 ml of 0.5 M Tris-HCl, 2.0 ml of 10% SDS, 0.2 ml of 0.5% W/V bromophenol blue, 50 µl of β-

mercaptoethanol and 2.5 µl of glycerol (Sigma-Aldrich)]. The mixture was mixed by vortex then centrifuged at a speed of 13,000 rcf for 1 minute at room temperature. The samples were heated in a water bath for 10 minutes at 60 °C. The samples were centrifuged again before loading in the gel. The electrophoresis tank was filled with electrophoresis buffer (1.5 g of Tris, 7.2 g of glycine and 0.5 g of SDS (Sigma-Aldrich) into 500 ml of distilled water). 5 µl of PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific) and the desired concentration of each sample were loaded on a 12% polyacrylamide gel. The electrophoresis tank was run for 20 min at 80 V and then 1 hour at 160 V. Once bromophenol blue reached the bottom of the gel, the power was turned off.

3.3.5.5 Blotting

An electrical current was used to transfer the protein from the polyacrylamide gel to ECL nitrocellulose membrane (GE Healthcare Life Sciences). The transfer method involves placing a membrane on top of the gel, and a stack of blotting papers on top of that. The entire stack was placed vertically in transfer buffer [200 ml of methanol into 750 ml of distilled water and 100 ml of stock transfer buffer which consists of: 144 g of glycine, 30 g of Tris and 2 g of SDS (Sigma-Aldrich), and 250 ml of distilled water (pH adjusted to 8.3)]. The transfer tank was connected to the power supply and electrophoresed at 58 mA for 2 hours on ice.

The membranes were then blocked with blocking agents (Marvel skimmed milk or BSA) for 1 hour at room temperature and incubated overnight at 4 °C with

suitably diluted primary antibodies. Membranes were washed three times with 0.5 % Tween PBS for 15 minutes. Membranes were incubated for 1 hour at room temperature with diluted horseradish peroxidise conjugated secondary antibody. Proteins were detected using an enhanced chemiluminescence (ECL) detection reagent (GE Healthcare Life Sciences). Each membrane was exposed to Amersham Hyper-film ECL (GE Healthcare Life Sciences) for an appropriate time (between 30 seconds and 10 minutes). Film was developed in Multigrade Paper Developer solution (Ilford), rinsed with water and fixed in Rapid Film and Paper Fixer solution (Ilford) for 2 minutes. The film was left to dry. The primary and secondary antibodies which were used are summarized in Table 13.

For accurate quantitative measurements for the protein, single-protein loading controls, such as β-actin, that should not change between samples were used in every run to show any technical error such as overloading or mis-loading of the protein. To obtained semi-quantitative analysis from three independent experiments, ImageJ software (National Institutes of Health) was used to quantify each blot by dividing the signal intensity of the protein of interest by the signal intensity of the internal control β-actin. Expression was classified as: 0; no expression, <0.5; low, 0.5-1; moderate, >1 high.

Primary antibody	Company	Secondary antibody	Company
[ab15459]: Rabbit polyclonal anti-β5.	Abcam	Polyclonal goat anti rabbit linked to horseradish peroxidise	Dako
[Q-20]: Rabbit polyclonal anti-αv.	Santa Cruz	Polyclonal goat anti rabbit linked to horseradish peroxidise	Dako
[B-7]: Mouse monoclonal anti-β3.	Santa Cruz	Polyclonal rabbit anti mouse linked to horseradish peroxidise	Dako
Mouse monoclonal antibody anti-β-actin.	Sigma Aldrich	Polyclonal rabbit anti mouse linked to horseradish peroxidise	Dako
[C-9]: Mouse monoclonal anti-α5.	Santa Cruz	Polyclonal rabbit anti mouse linked to horseradish peroxidise	Dako

Table 13 Primary and secondary antibodies used in Western blot studies for detection of αv, β5, α5 and β3 integin subunits.

3.3.6 Immunohistochemistry

3.3.6.1 Frozen tissues

Before cutting sections, blocks were equilibrated to the cryostat temperature (−20 °C) for 24 hours. The blocks were sectioned to 5 µm thickness using cryostat (Leica CM1100, Leica Microsystems, Nussloch Germany). Sections were picked up onto 3-aminopropyltriethoxysilane (APES) coated slides and left at room temperature till the sections were adhered to the slides. Slides were stored at −80 °C until staining.

3.3.6.1.1 **Haematoxylin and eosin staining of frozen tissue**

Tissue sections were stained as described in section 2.3.2.

3.3.6.1.2 **Immunohistochemical labelling of frozen sections**

Immunohistochemical labelling of frozen sections was carried out as described in section 2.3.3. The primary and secondary antibodies which were used are summarized in Table 14.

3.3.6.1.3 **Mouse on mouse (M.O.M.) kit**

For antibodies raised from mouse, an M.O.M kit (Vector Laboratories) was used to localise mouse primary antibodies on mouse tissues which should eliminate background staining in mouse tissue. The 5 µm thick frozen tissues were fixed in acetone for 10 minutes and then washed with PBS for 10 minutes. The endogenous enzyme activity was blocked with bloxall endogenous peroxidase and alkaline phosphatase blocking solution (Vector Laboratories) for 10 minutes. After washing 10 minutes with PBS, sections were blocked for one

hour with M.O.M. mouse Ig blocking reagent (90 µl of stock solution with 2.5 ml of PBS) and incubated for 5 minutes with working solution of M.O.M diluent (600 µl of protein concentrate stock solution to 7.5 ml of PBS). Sections were incubated for 30 minutes with diluted primary antibody and then washed three times with PBS and incubated for 10 minutes with M.O.M. biotinylated anti-mouse IgG (10 µl of stock solution to 2.5 ml of prepared M.O.M. diluent). Tissue sections were then processed as described in section 2.3.3.

3.3.6.2 Paraffin embedded tissues

Tumours were immersed in 10% neutral buffered formalin (VWR International Ltd. Poole, UK) for 24 to 48 hours and then placed in plastic cassettes (Raymond A. Lamb Ltd, UK). The sample was then placed in an automatic tissue processor (Leica TP 1020, Leica Microsystems, Nussloch, Germany) where the samples transferred through baths of progressively more concentrated ethanol (70 %, 80 %, 95 % and 100%) and xylene to clear any remaining ethanol. Fixed tissues were then embedded in the wax and allowed to harden overnight. After that the blocks were sectioned to 5 µm thickness using a Leitz rotary microtome (Leica RM2155, Leica Microsystems, Nussloch, Germany). Sections were attached onto 3-aminopropyltriethoxysilane (APES) coated slides and stored at room temperature.

3.3.6.2.1 Haematoxylin and eosin staining of paraffin embedded tissues

Tissue sections were de-waxed and rehydrated by immersing twice in different tank of 100% xylene for 10 minutes, once in 50% xylene/ethanol for 5 minutes,

twice in absolute ethanol for 10 minutes, once in 90% ethanol for 5 minutes, once in 70% ethanol for 5 minutes and finally in distilled water for 5 minutes. Sections were then stained as described section 2.3.2.

3.3.6.2.2 **Immunohistochemical staining of paraffin embedded tissues**

Tissue sections were de-waxed and rehydrated as described in section 3.3.6.2.1. To block the endogenous peroxidase activity, tissues were treated with 1% hydrogen peroxide (Vector Laboratories) for 30 minutes and then rinsed 10 minutes in PBS. For antigen retrieval, slides were placed in a plastic container of citrate buffer (2.1 g citric acid monohydrate in 950 ml of distilled water) and heated by the microwave at 600 W for 20 minutes or tissues were covered by pre-warmed trypsin (400 mg trypsin + 8 ml of 5% $CaCl_2$ in 400 ml of distilled water) for 15 minutes at 37 °C. Following another PBS wash, the sections were circled with a wax pen and stained as described in section 2.3.3.

Primary antibody	Company	Secondary antibody	Company
[ab15459]: Rabbit polyclonal anti-β5.	Abcam	Biotinylated goat anti-rabbit IgG antibody	Vector labs
[Q-20]: Rabbit polyclonal anti-αv.	Santa Cruz	Biotinylated goat anti-rabbit IgG antibody	Vector labs
[B-7]: Mouse monoclonal anti-β3.	Santa Cruz	Biotinylated horse anti-mouse IgG antibody	Vector labs
[C-9]: Mouse monoclonal anti-α5.	Santa Cruz	Biotinylated horse anti-mouse IgG antibody	Vector labs

Table 14 Primary and secondary antibodies used in immunohistochemistry studies for detection of αv, β5, α5 and β3 integin subunits in xenograft tissues.

3.4 Results

3.4.1 Protein expression in cell lines

3.4.1.1 Immunofluorescence

3.4.1.1.1 **αv integrin subunit**

The optimal conditions for using each antibody for immunofluorescence are summarized in Table 15. The Q-20 antibody was used to determine the expression and the localization of αv integrin subunit on a panel of cell lines comprising: MCF-7, MCF-7ADR, DLD-1, PC-3, DU-145, HT-29, HUVEC and OSC-19. The MCF-7 cell line was used to optimize the Q-20 antibody because literature suggested it expresses a high level of αv integrin subunits (Taherian et al., 2011). The steps followed to optimize the αv antibody (Q-20) are summarized in Figure 19**.** The optimized conditions were: fixation with 4% paraformaldehyde, blocked with 5% BSA with 0.3 M glycine, overnight incubation at 4 °C with (1:50) primary antibody (Q-20) and 1 hour incubation at room temperature with (1:200) diluted secondary antibody (Alexa Fluor 546 donkey anti rabbit IgG).

The immunofluorescence images suggest localisation of the αv integrin subunit on the cell membrane. The αv integrin subunit screening results showed that all these cell lines express the αv integrin subunit, but with variable levels. PC-3 has very weak expression, whereas the OSC-19, DLD-1, HT-29 and HUVEC cells have the highest expression. The DU145, MCF-7 and MCF-7ADR cell lines demonstrated low expression (see Figure 20).

	Anti αv	Anti β5	Anti α5 (c-9)
Fixation	4% PFA	Acetone	Acetone
Blocking	5% BSA/PBS	5% BSA/PBS	5% BSA and 10 goat serum
Primary antibody dilution	1:50	1:200	1:100
Incubation condition	Overnight at 4 °C	One hour at room temperature	Overnight at 4 °C
Secondary antibody dilution	1:200 for 1 hour in dark.	1:200 for 1 hour in dark.	1:200 for 1 hour in dark.

Table 15 Summary of optimal conditions for each antibody in immunofluorescence

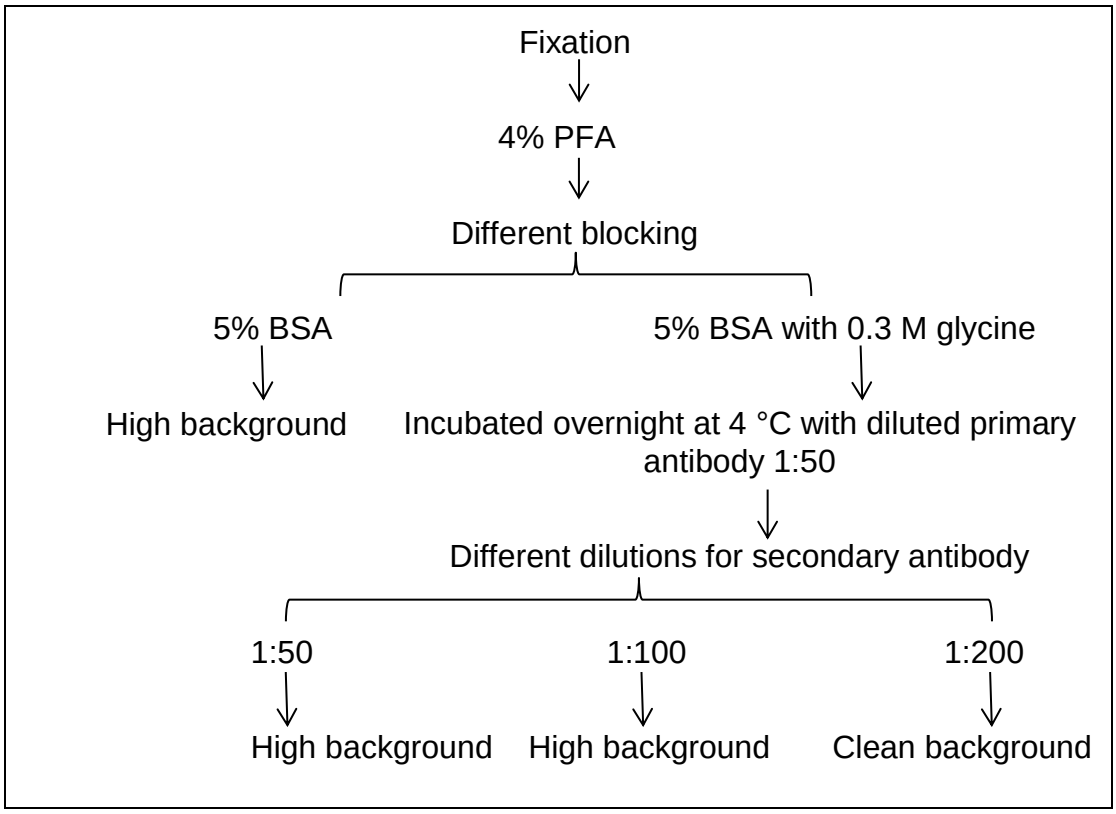


Figure 19 The steps followed to optimize the αv antibody (Q-20).

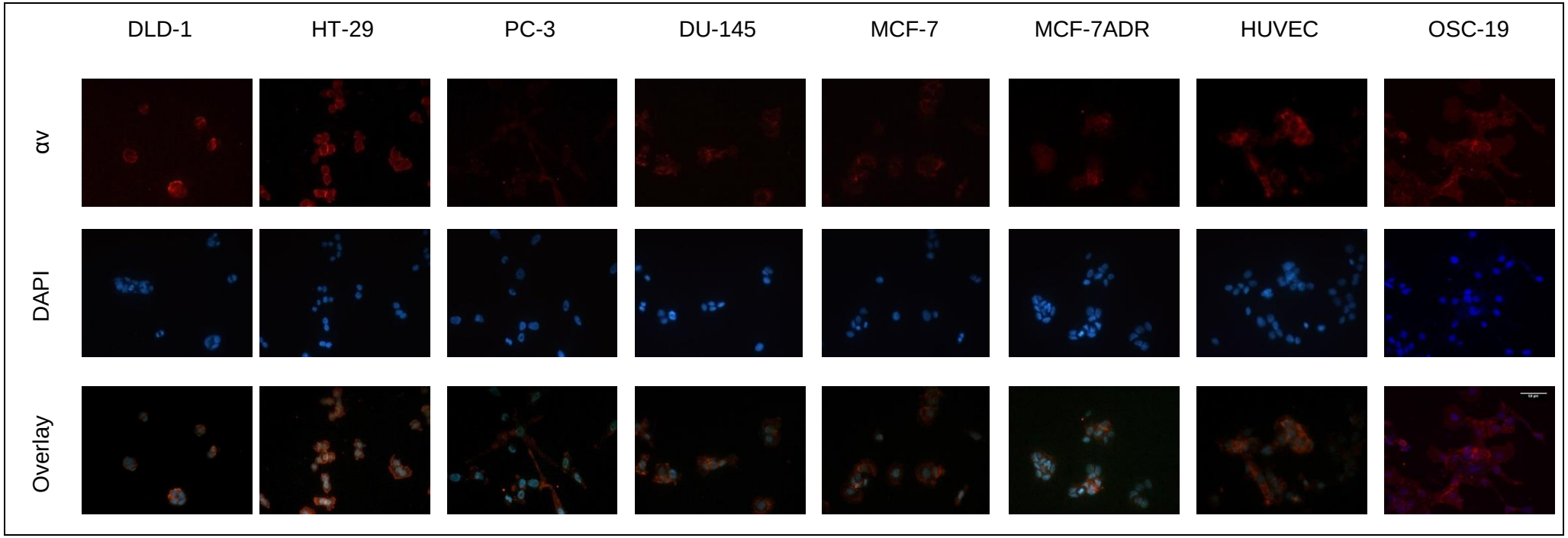


Figure 20 Immunofluorescence images of αv expression in cancer cell lines.

DAPI stained the nuclei (blue) and the Q-20 antibody labelled αv integrin subunit, which may have been localised on the cell membrane (red). Scale bar = 50 μm.

3.4.1.1.2 **β5 integrin subunit**

The ab15459 antibody was used to determine the expression of the β5 integrin subunit. The MCF-7 cell line was used to optimize this antibody because literature suggested it expresses a high level of β5 integrin subunits (Taherian et al., 2011). The steps followed to optimize the β5 antibody (ab15459) are shown in Figure 21.

MCF-7 Cells were fixed with acetone and blocked with 5% BSA/PBS. The cells were incubated 1 hour at room temperature with primary antibody (ab15459) (1:200) and 1 hour at room temperature with diluted secondary antibody (Alexa Fluor 546 donkey anti rabbit IgG) (1:200). The immunofluorescence images suggest the localisation of the β5 integrin subunit in the cytoplasm and cell membrane. The β5 integrin subunit screening results showed that all cell lines express β5, but at variable levels. As shown in Figure 22, DU-145 has the lowest expression, while OSC-19, MCF-7ADR, PC-3, DLD-1 and MCF-7 have the highest. HT-29 and HUVEC have moderate levels of the β5 integrin subunit.

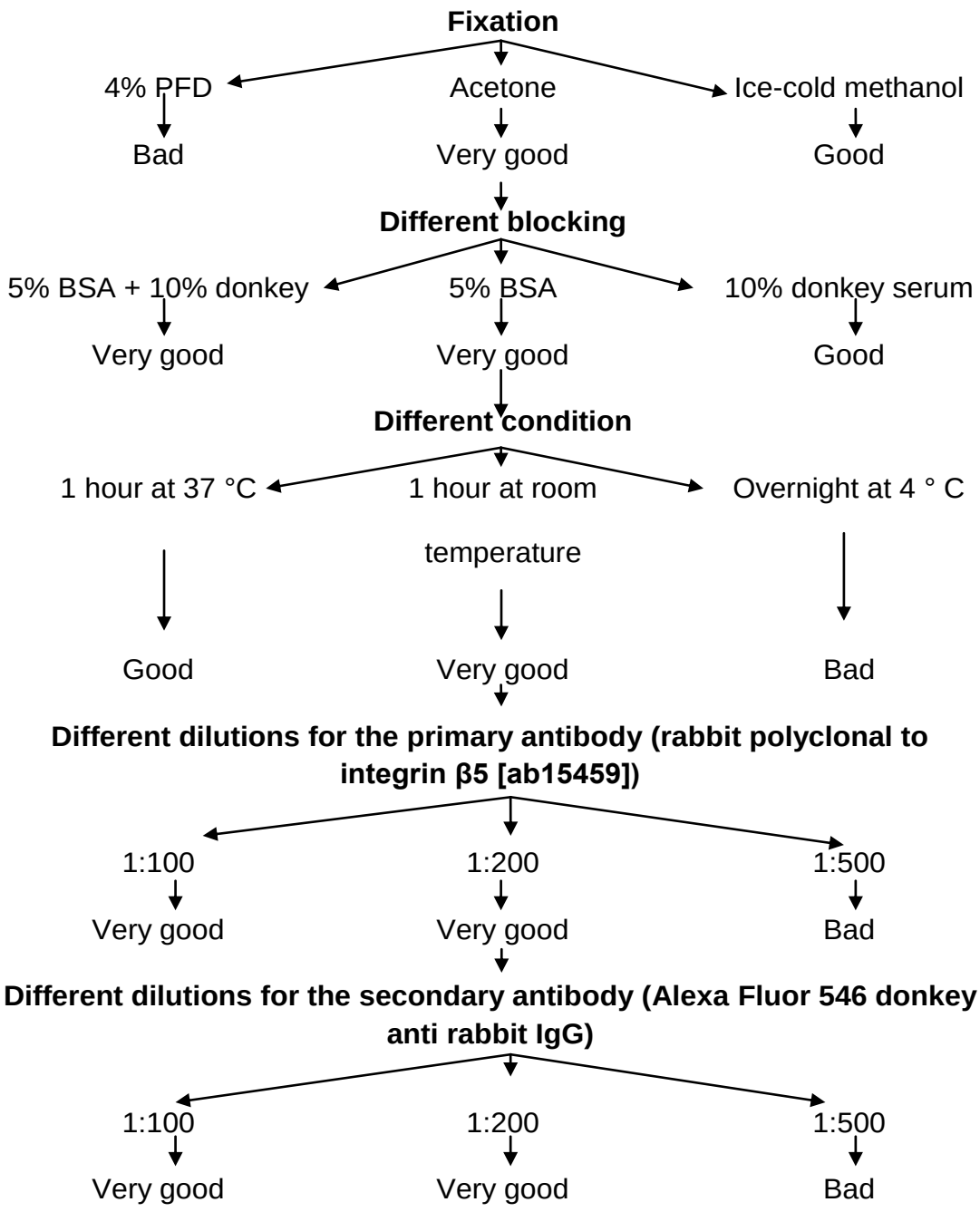


Figure 21 The steps followed to optimize the β5 antibody (ab15459).

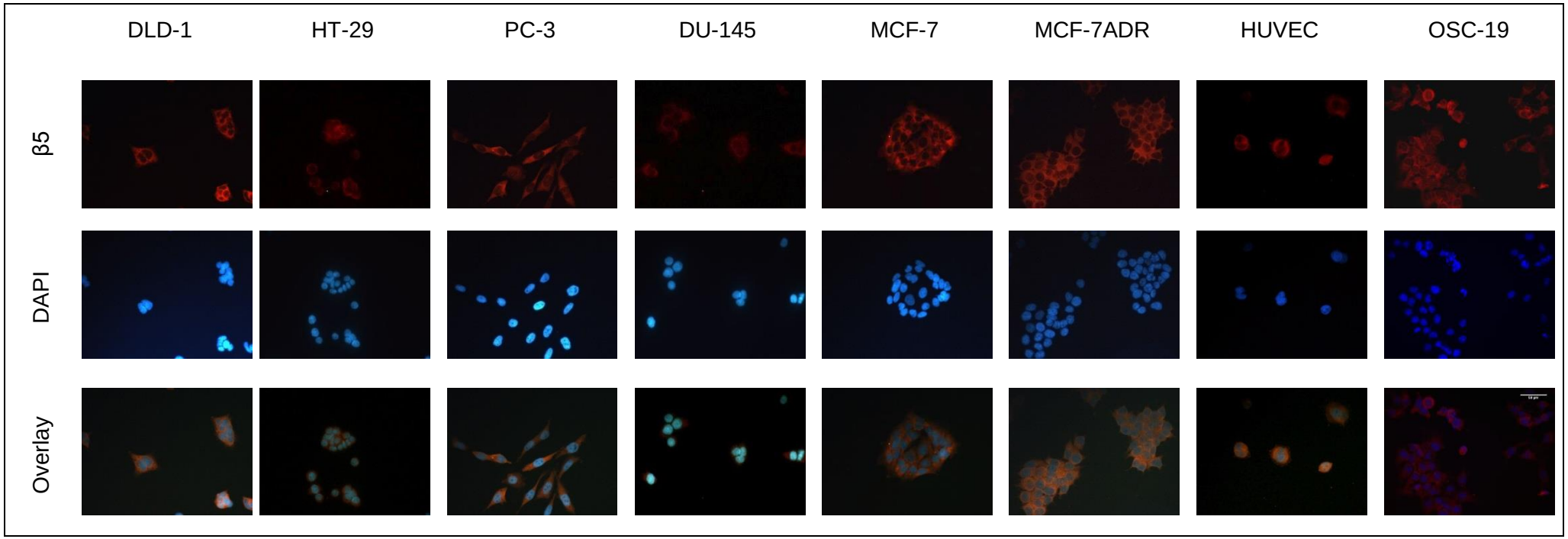


Figure 22 Immunofluorescence images of β5 expression in cancer cell lines.

DAPI stained the nuclei (blue) and ab15459 antibody labelled β5 integrin subunit, which may have been localised on the cell membrane and in the cytoplasm (red). Scale bar = 50 μm.

3.4.1.1.3 **α5 integrin subunit**

The C-9 antibody was used to determine the expression of the α5 integrin subunit. The MCF-7ADR cell line was used to optimize this antibody because literature suggested it expresses a high level of α5 integrin subunits (Nista et al., 1997). The steps followed to optimize the α5 antibody are shown in Figure 23.

MCF-7ADR cells were fixed with acetone and blocked with mixture containing 5% BSA and 10% goat serum. The cells were incubated overnight at 4 °C with diluted primary antibody (1:100) and 1 hour at room temperature with diluted secondary antibody (Alexa Fluor 488 F(ab)2 fragment of goat anti mouse) (1:200). The immunofluorescence images show that the expression of the α5 integrin subunit is potentially localised to the cell membrane and cytoplasm in the MCF-7ADR, DLD-1, PC-3, DU-145, HUVEC and OSC-19 cell lines. The HT-29 and MCF-7 cell lines do not express the α5 integrin subunit (Figure 24).

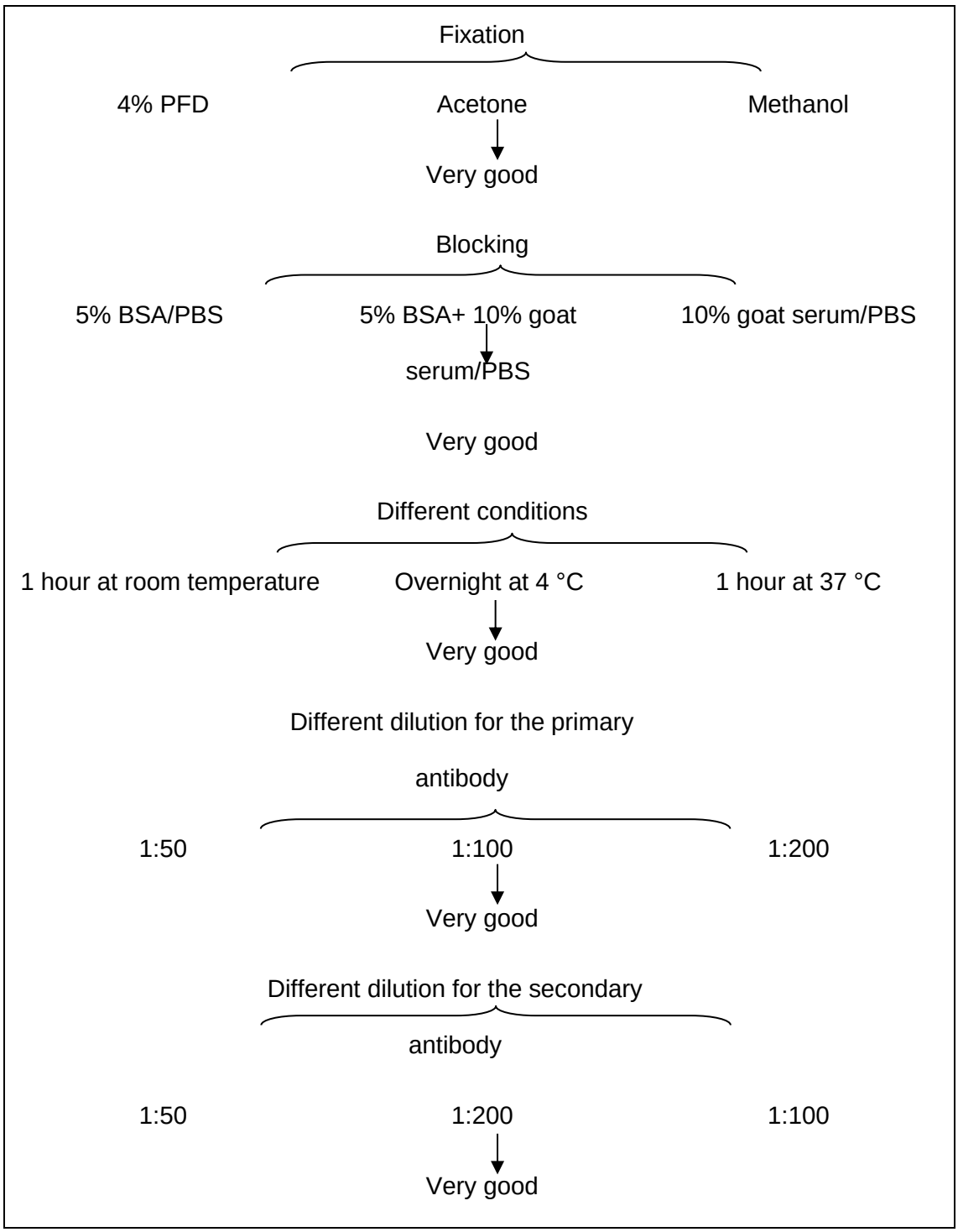


Figure 23 The steps followed to optimize the α5 antibody (C-9).

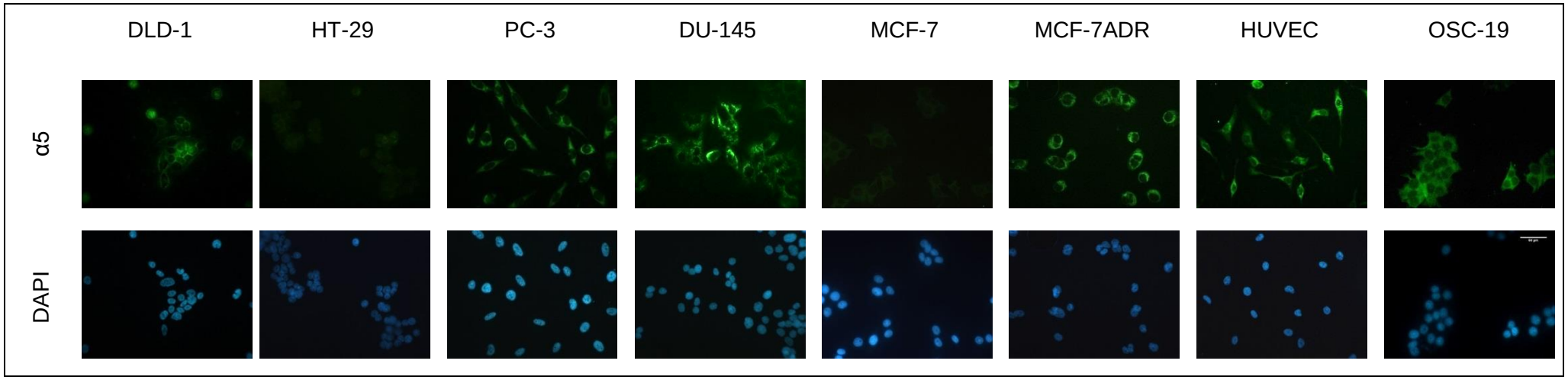


Figure 24 Immunofluorescence images of α5 expression in cancer cell lines.

DAPI stained the nuclei (blue) and C-9 antibody labelled α5 integrin subunit, which may have been localised on the cell membrane and in the cytoplasm (green). Scale bar = 50 μm.

3.4.1.2 Western blot on cell line

Whilst immunofluorescence provided evidence of spatial localisation of the integrin proteins, the results were not quantitiative i.e they did not demonstrate relative amounts of protein present or specificity of the antibody for a specific integrin; for this purpose Western blotting was used. The optimum conditions for each antibody are summarised in Table 16.

3.4.1.2.1 αv integrin subunit

All cell lines (MCF-7, MCF-7ADR, DLD-1, PC-3, DU-145, HT-29, HUVEC and OSC-19) expressed αv but the relative expression profile differed between cell lines. HUVEC, OSC-19, M14, DLD-1 and HT-29, expressed a moderate αv level of the integrin subunit, whereas others, such as MCF-7ADR, MCF-7 and DU-145, expressed low αv levels and PC-3 cells did not express αv (Figure 27).

3.4.1.2.2 β5 integrin subunit

The process used to optimize β5 antibody is explained in Figure 25. β5 integrin was expressed at high levels by DU145, M14, OSC-19, DLD-1 and MCF-7 and at moderate level by HT-29, HUVEC and MCF-7ADR. PC-3 cells expressed low level of β5 integrin subunit (Figure 27).

3.4.1.2.3 α5 integrin subunit

The optimisation of the α5 antibody is detailed in Figure 26. DLD-1, DU145, MCF-7ADR, HUVEC, OSC-19 and M14 expressed a high level and PC-3 expressed moderate level of the α5 integrin subunit. HT-29 and MCF-7 cells did not express α5 integrin subunit (Figure 27).

	Anti αv	Anti β5	Anti α5	Anti-β actin
Lysis buffer to extract protein from cells	Triton	Urea	Urea	Triton or Urea
Lysis buffer to extract protein from xenograft tissues	Riba + Urea	Riba + Urea	Riba + Urea	Riba + Urea
Blocking	5% BSA/PBS	5% milk	5% milk	5% BSA/PBS
Primary antibody dilution	1:2000	1:2000	1:300	1:2000
Incubation condition	Overnight at 4 °C	Overnight at 4 °C	Overnight at 4 °C	Overnight at 4 °C
Secondary antibody	1:3000	1:2500	1:3000	1:3000
Exposure time	10 min	10 min	10 min	30 sec
Expected result	Single band size 130 KDa	Single band size 85 KDa	Single band size 150 KDa	Single band size 44 KDa

Table 16 Summary of optimal conditions for each antibody in Western blot

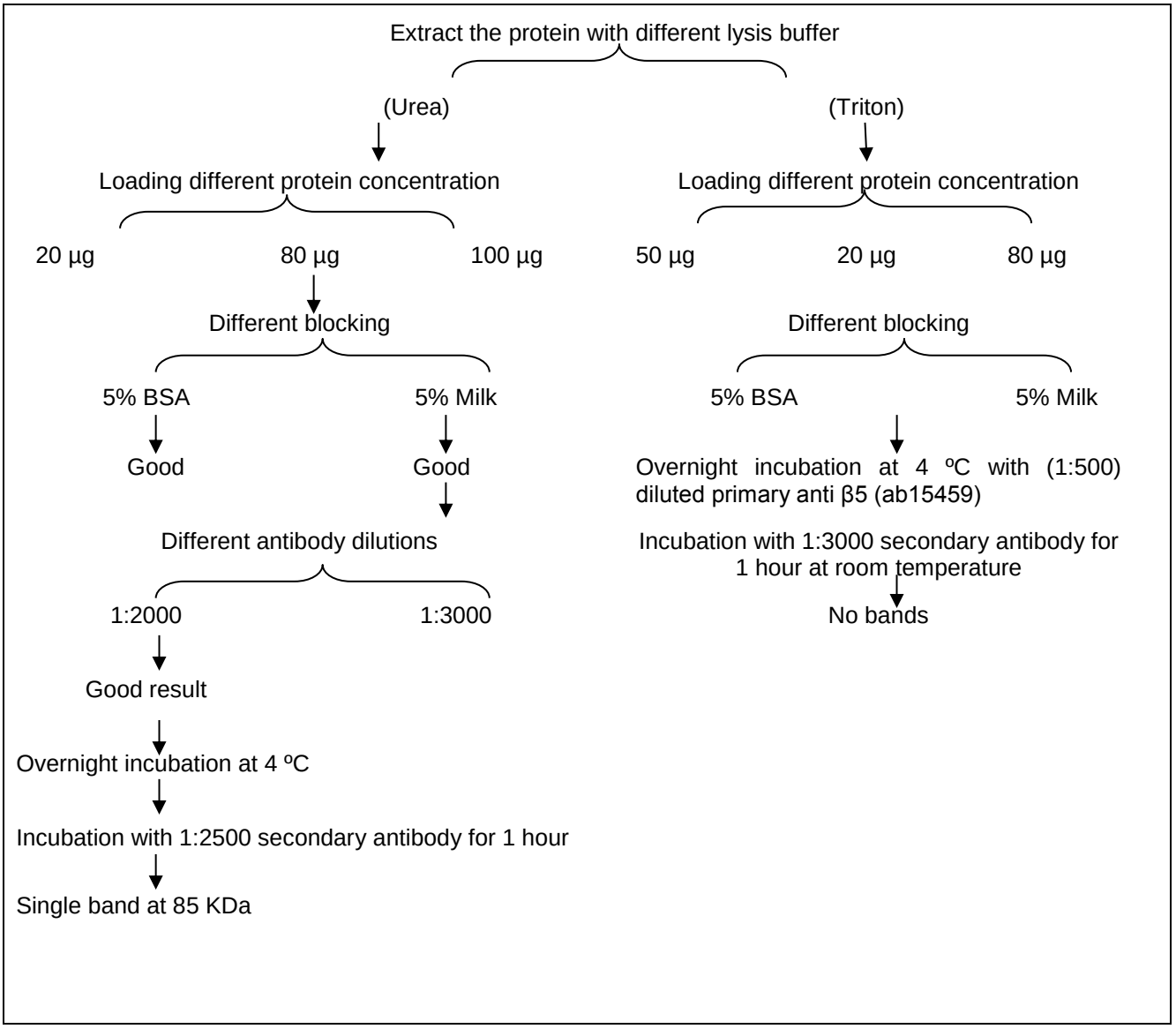


Figure 25 The steps followed to optimize the β5 antibody (ab15459).

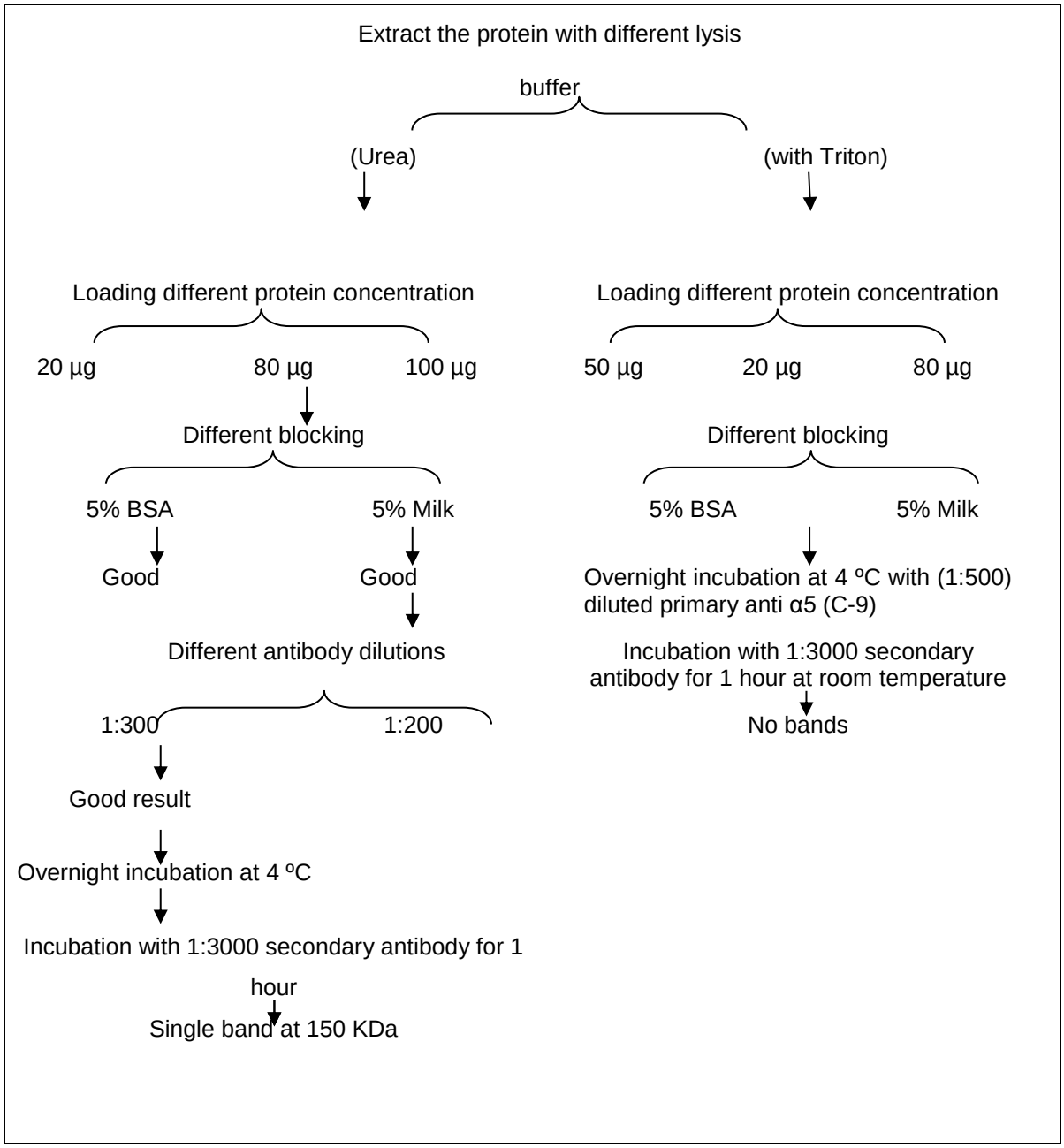


Figure 26 The steps followed to optimize the α5 antibody (C-9).

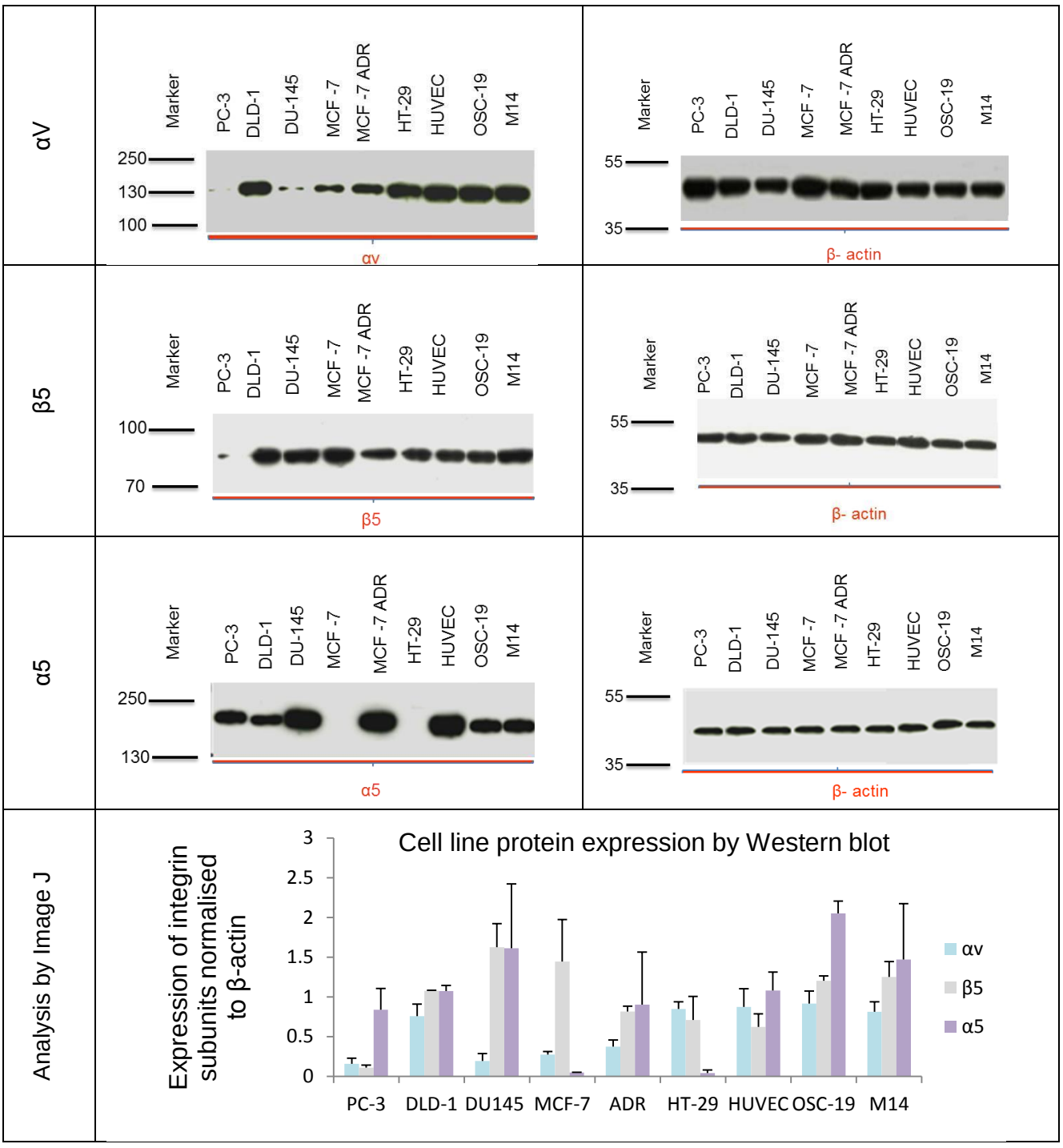


Figure 27 Expression of αv, β5 and α5 integrin subunits in a panel of human tumour cell lines by Western blot.

Membranes were blotted with anti αv (B-Q), anti β5 (ab15459), anti α5 (C-9) and anti β-actin antibodies, which recognize proteins with band size 130, 85, 150 and 42 kDa respectively. Results shown are the average of three independent experiments.

3.4.2 Protein expression in xenograft tissues

3.4.2.1 IHC on xenograft tissues

The steps which were followed to optimize the antibodies are shown in Figure 28. The optimal conditions for each antibody used in immunohistochemistry are summarized in Table 17. To evaluate the expression of αv, β3 α5 and β5 integrin subunits on xenograft tissues, two rabbit polyclonal antibodies, Q20 (anti-αv) and ab15459 (anti-β5) and two mouse monoclonal antibodies, B7 (anti-β3) and C-9 (anti-α5) were used. On formalin fixed paraffin embedded (FFPE) tissue, the four antibodies did not label the protein, most likely because the FFPE process involves cross-linking, dehydration, hydrophobic environments, and heat, all of which can destroy or conceal epitopes (Figure 29). On fresh frozen xenograft tissues, strong cytoplasmic immunolabelling without non-specific background was observed with rabbit antibodies against αv (Q-20) and β5 (ab15459). Non-specific labelling of mouse tissues with mouse antibodies made it difficult to identify α5 and β3 integrin subunit distribution on mouse xenografts. A M.O.M kit was used to prevent non-specific binding but unfortunately all methods proved unsuccessful (Figure 30). Therefore, the expression of α5 and β3 integrin subunits was evaluated by Western blot (Figure 32).

On HT-29 and MCF-7 xenografts, αv integrin subunit was intensely expressed only on tumour cells whereas the β5 integrin subunit was moderately expressed (Figure 31). On the OSC-19 xenograft, αv and β5 integrin subunits were highly

expressed on tumour cells and stroma respectively (Figure 31). On the PC-3 xenograft, both tumour cells and stroma expressed moderate level of αv and β5 integrin subunits (Figure 31).

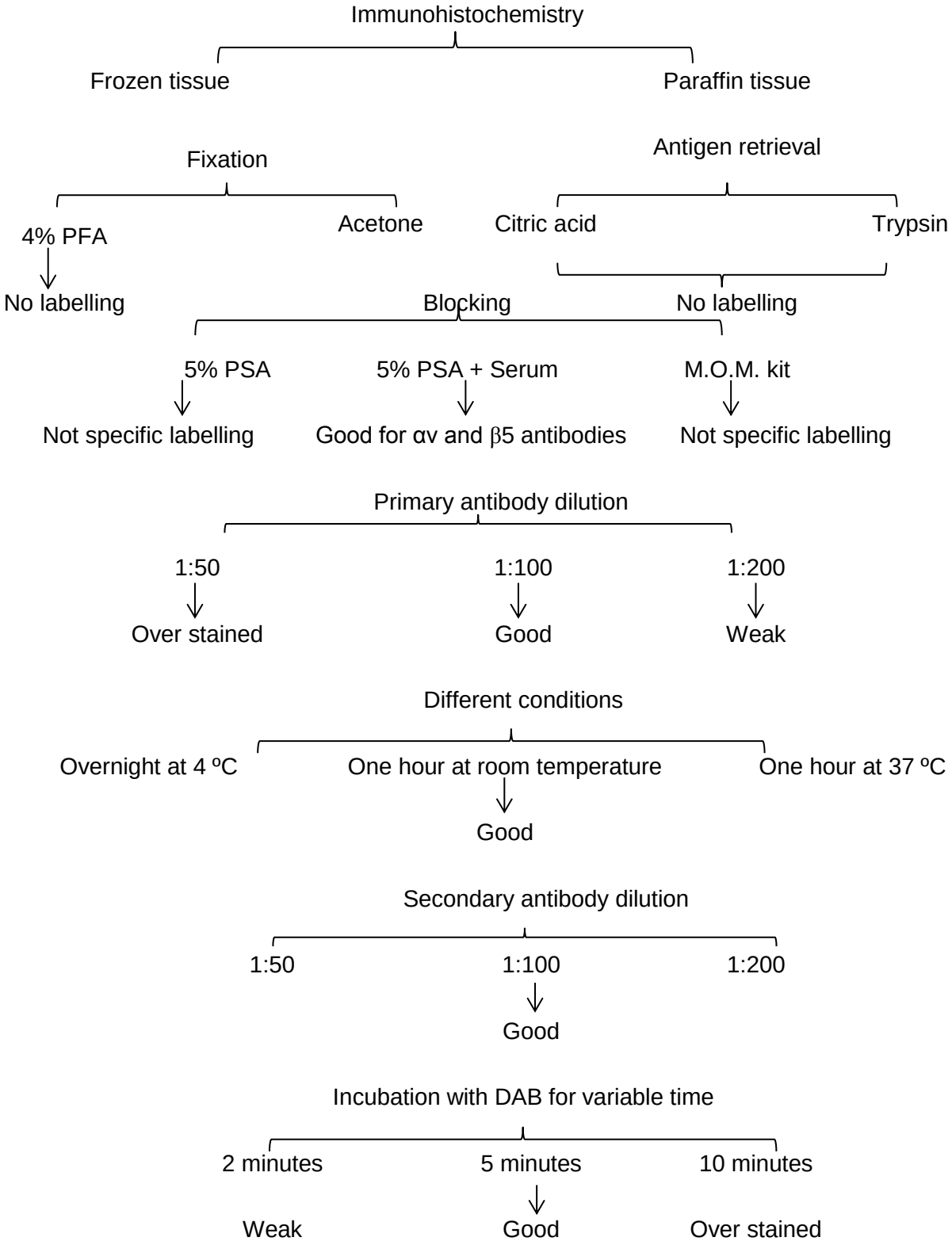


Figure 28 The steps which were followed to optimize the antibodies in immunohistochemistry

Antibody	Q-20 and ab15459
Type of tissue	Frozen
Fixation	Acetone (10 minutes)
Blocking	5% BSA/PBS + 10 serum
Primary antibody dilution	1:100
Incubation condition	One hour at room temperature
Secondary antibody	1:200
DAB	5 min

Table 17 Summary of optimal conditions for rabbit antibodies in immunohistochemistry

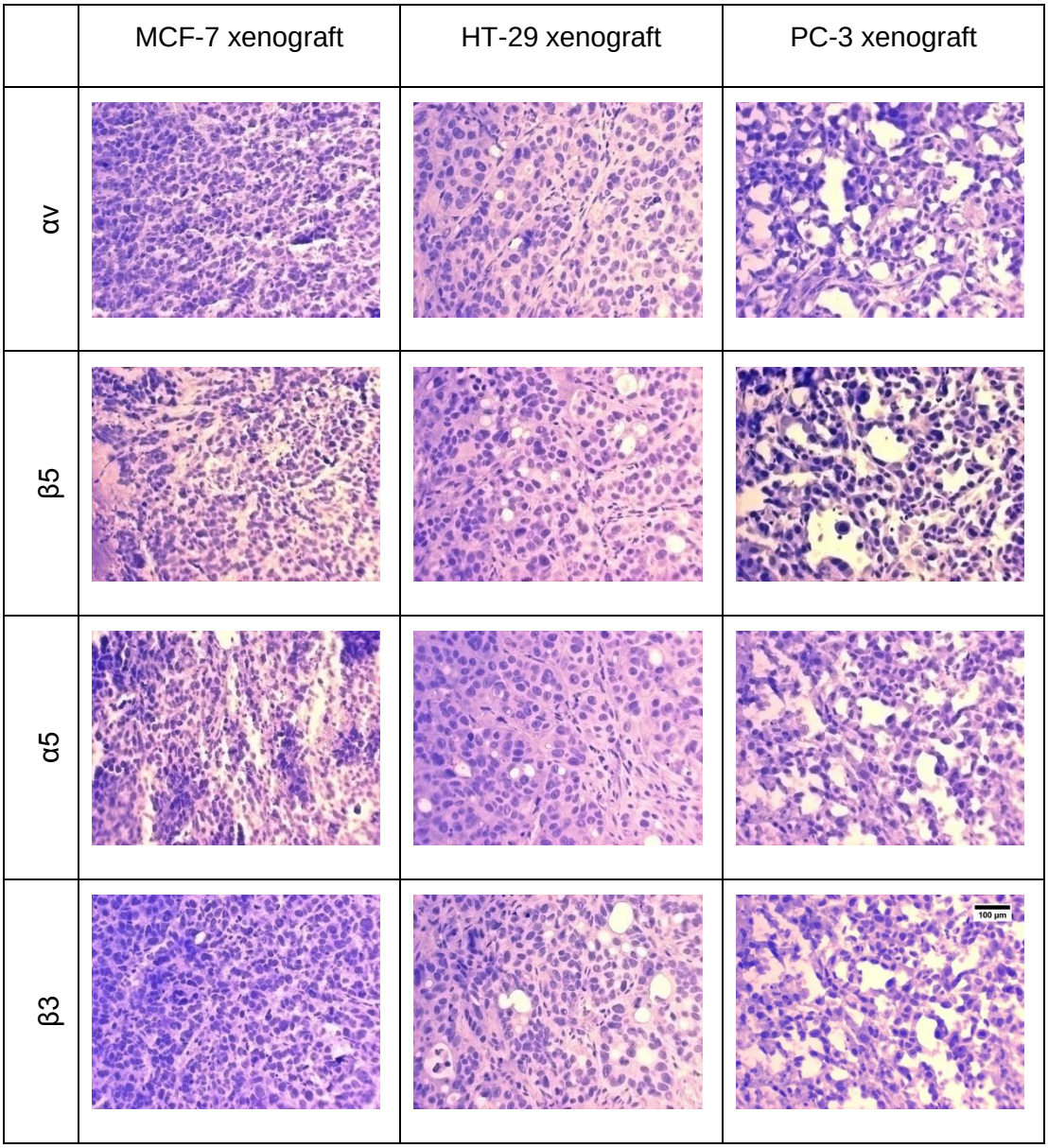


Figure 29 αv, β5, α5 and β3 integrin subunit detection in FFPE MCF-7, HT-29 and PC-3 xenografts.

IHC was unsuccessful for immunodetection of αv, β5, α5 and β3 integrin subunits in FFPE tissue using Q20 (anti-αv), ab15459 (anti-β5), B7 (anti-β3) and C-9 (anti-α5). No labelleling was observed. Scale bar = 100 µm.

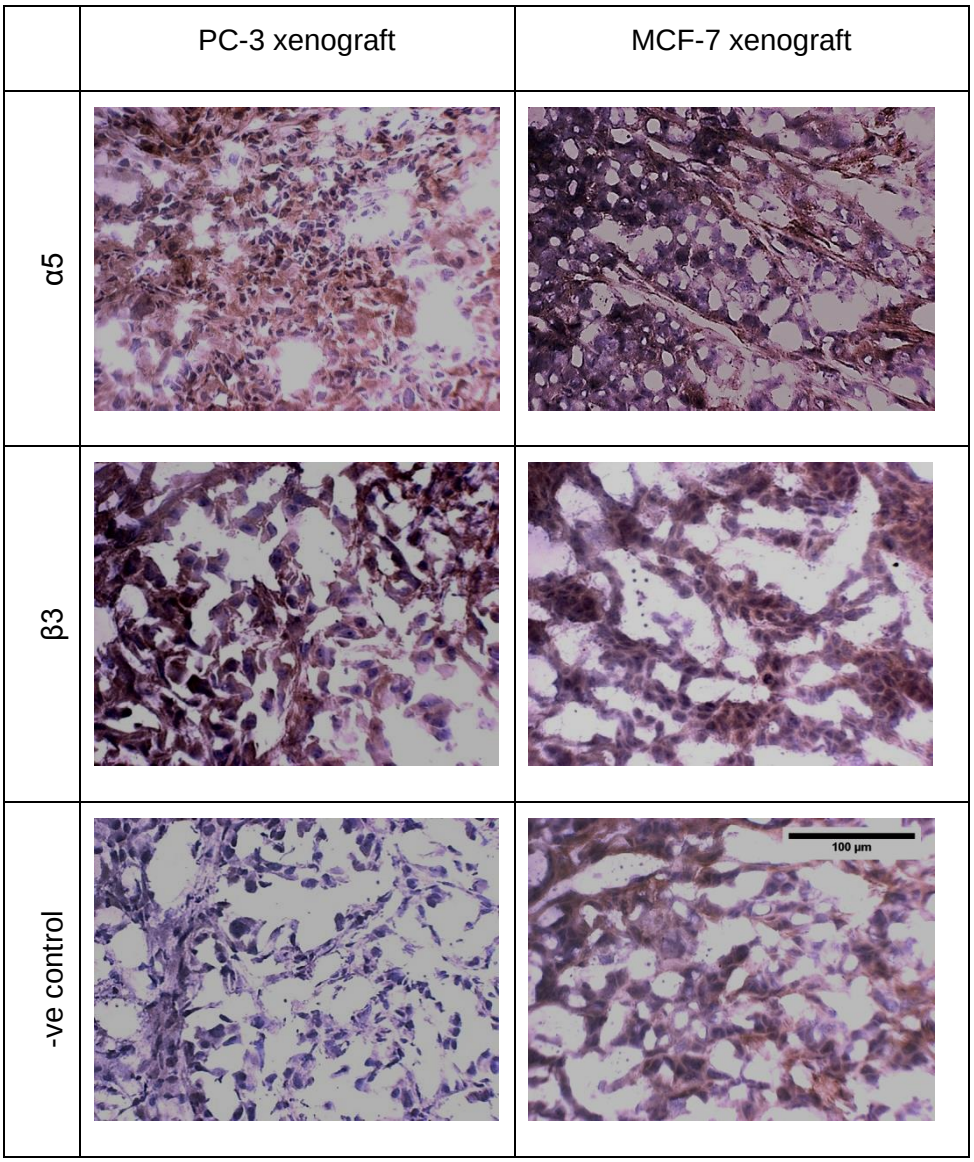


Figure 30 Immunohistochemistry with mouse antibodies on fresh-frozen xenograft material.

A M.O.M kit was used to detect the expression of α5 and β3 integrin subunits in fresh frozen MCF-7 and PC-3 xenografts by using mouse antibodies B7 (anti-β3) and C-9 (anti-α5). Unspecific labelling of mouse tissues with mouse antibodies was observed in the negative control. Scale bar = 100 µm. Scale bar = 100 µm.

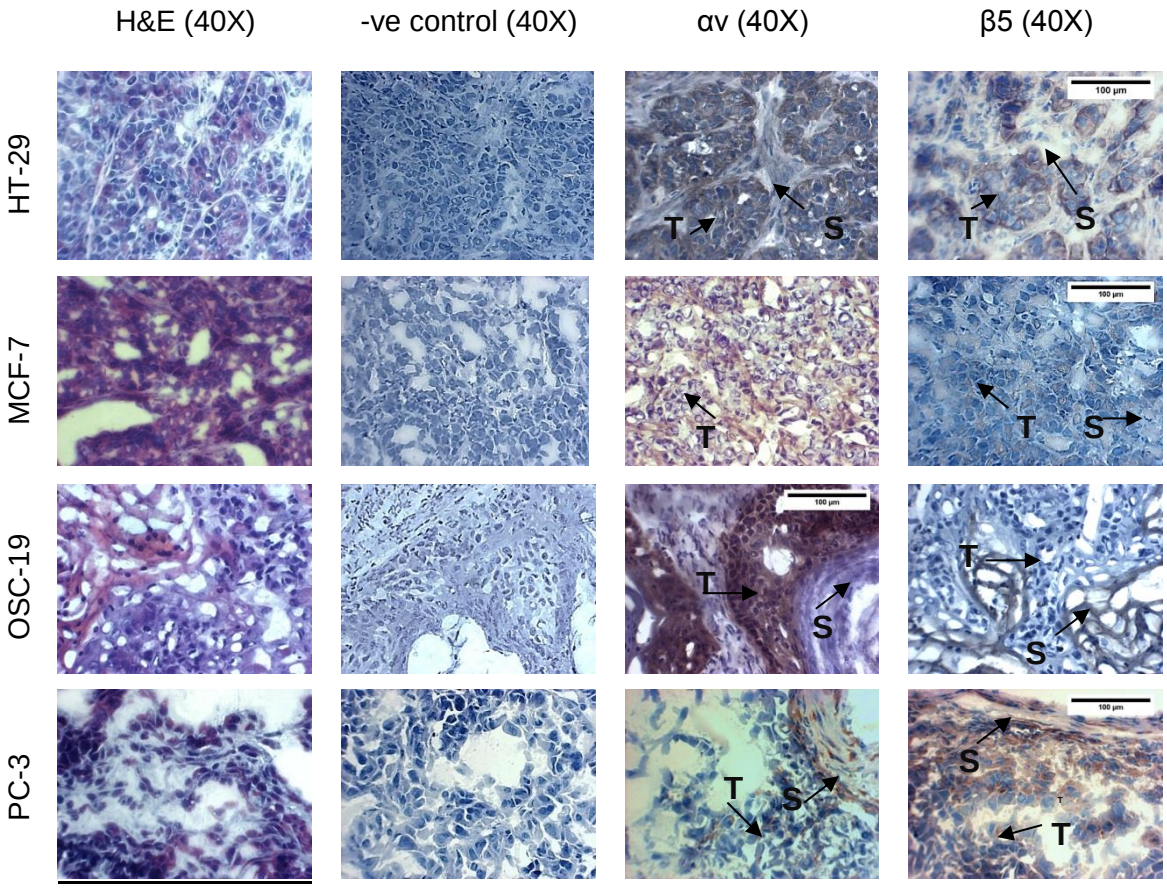


Figure 31 Expression of αv and β5 subunits in fresh frozen xenografts using rabbit antibodies.

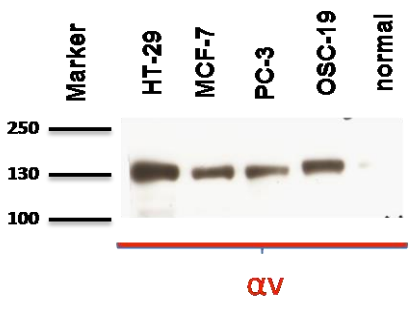
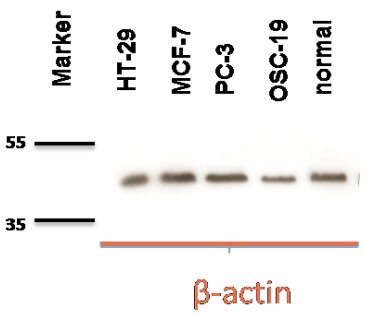
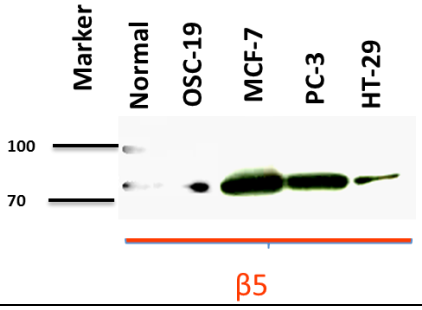
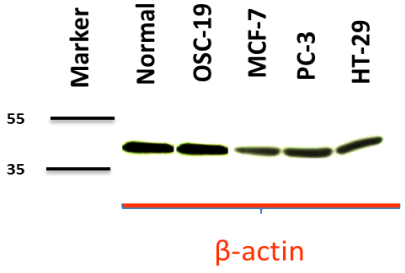
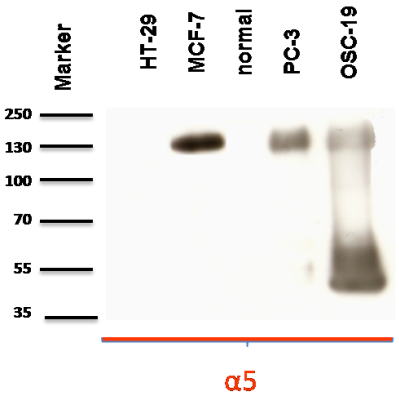
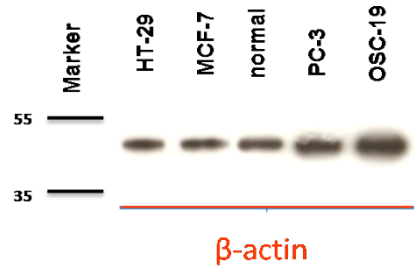
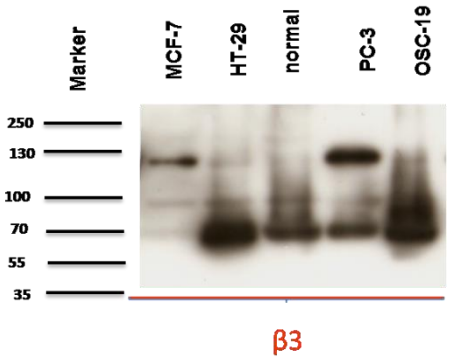
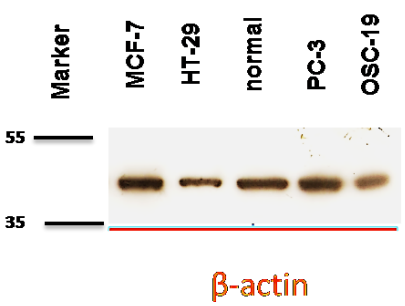
Q-20 and ab15459 antibodies gave cytoplasmic staining. -ve control which was stained with secondary antibody only was clean. T; tumour cells, S; stroma. Scale bar = 100 µm.

3.4.2.2 Western blot of xenograft tissues

Western blot results showed that Q-20 anti-αv antibody bound to a protein of weight circa 130 KDa in all xenograft tissues. OSC-19 and HT-29 xenografts expressed the αv integrin subunit strongly, whereas PC-3 and MCF-7 xenografts expressed αv moderately. The anti-β5 antibody (Ab15459) gave a single band on 85 KDa in all xenografts. The β5 integrin subunit was highly expressed on PC-3, MCF-7 and HT-29 xenografts and weakly on OSC-19 xenograft. The anti-β3 antibody (B7) gave two bands; one band at 120 kDa consistent with the β3 integrin, and another band, at circa 60 kDa. The lower molecular weight bands resulted from using secondary anti-mouse antibody on mouse tissues since it was also obtained when the secondary antibody was added to membrane without primary antibody (Figure 32). The β3 integrin subunit was moderately expressed on MCF-7 and OSC-19 xenografts whereas its expression was low in HT-29 xenografts. PC-3 xenografts expressed the highest amount of β3 integrin subunit. The anti-α5 antibody (C-9) recognised two protein bands; one at the molecular weight, 150 kDa consistent with α5 integrin and one band at about 60 kDa. The lower molecular weight bands resulted from using secondary anti-mouse antibody on mouse tissues since it was also obtained when the secondary antibody was added to membrane without primary antibody. The MCF-7 xenograft had the highest expression of α5 integrin subunit; whereas PC-3 and OSC-19 xenografts had a moderate level of α5 integrin subunit. Normal mouse tissue showed no protein bands that were detected by the human integrin antibodies but a faint signal in the normal

lane on β5 blot, this mean that ab15459 antibody binds with mouse integrin as well (Figure 32 and Figure 33).

The expression of αv, β3, β5 and α5 integrin subunits was increased in most xenograft tissue compared with the corresponding cultured cells except the expression of β5 and α5 integrin subunits was elevated in OSC-19 cultured cells. The difference between the expression of αv integrin subunit in HT-29, PC-3 and MCF-7 xenograft tissue and corresponding cultured cells and β5 integrin subunit in OSC-19 cultured cells and corresponding xenograft tissue was statistically significant (P= 0.04, 0.03, 0.01 and 0.03 respectively) (Figure 34 and Table 18).

αV	 Marker HT-29 MCF-7 PC-3 OSC-19 normal 250 130 100 αv	 Marker HT-29 MCF-7 PC-3 OSC-19 normal 55 35 β-actin
β5	 Marker Normal OSC-19 MCF-7 PC-3 HT-29 100 70 β5	 Marker Normal OSC-19 MCF-7 PC-3 HT-29 55 35 β-actin
α5	 Marker HT-29 MCF-7 normal PC-3 OSC-19 250 130 100 70 55 35 α5	 Marker HT-29 MCF-7 normal PC-3 OSC-19 55 35 β-actin
β3	 Marker MCF-7 HT-29 normal PC-3 OSC-19 250 130 100 70 55 35 β3	 Marker MCF-7 HT-29 normal PC-3 OSC-19 55 35 β-actin

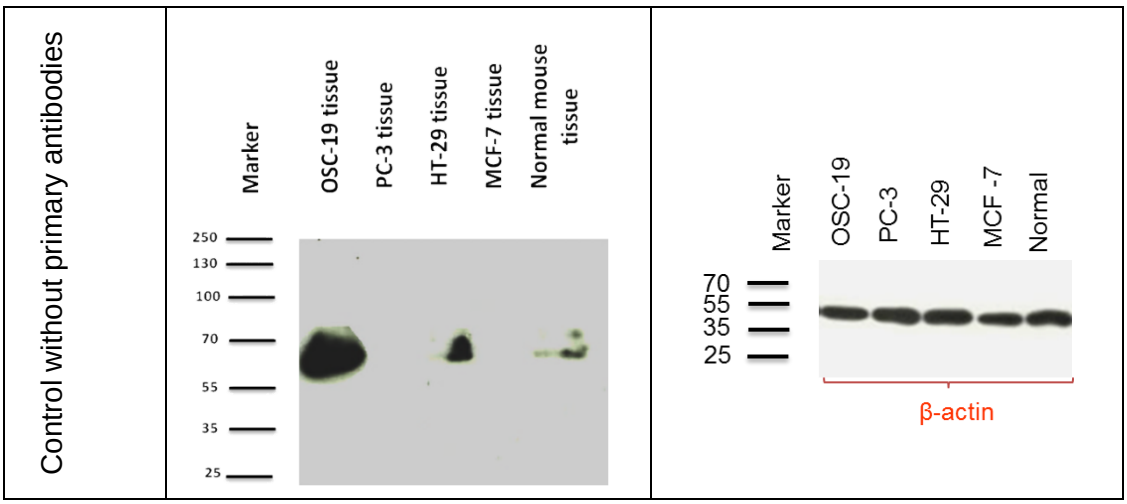


Figure 32 Integrin subunit expression on xenograft tissues by Western blot.

Mouse antibodies C-9 (α5) and B-7 (β3) gave two bands; one band at 120 kDa and another band, at circa 60 kDa whereas rabbit antibodies Q-20 (αv) and ab15459 (β5) antibodies gave specific single band at 130 kDa and 80 kDa respectively, in the control, the membrane was incubated with secondary antibody only and it gave single band at about 60 kDa.

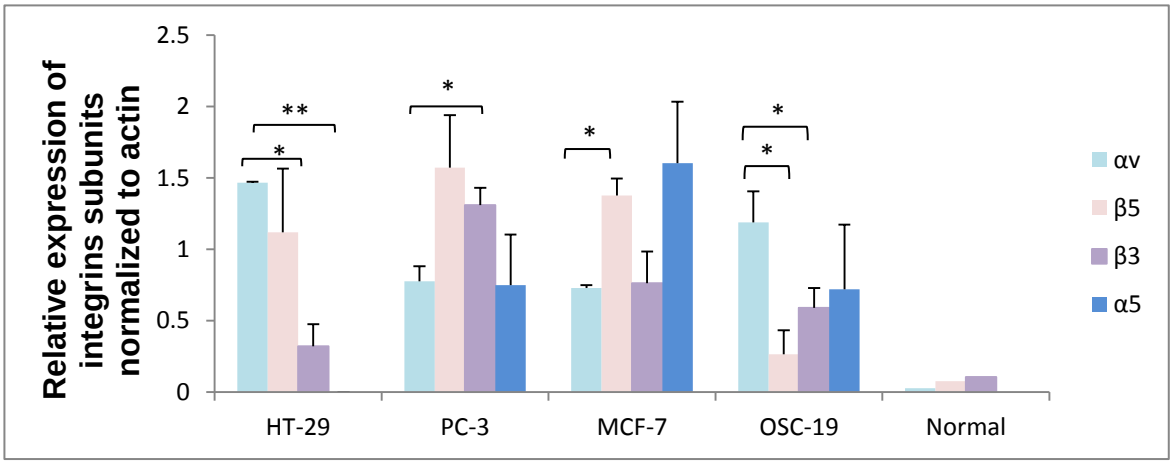


Figure 33 The expression of αv, β5, β3 and α5 integrin subunits in a panel of xenografts.

Results shown are the average of three independent experiments. * indicates $P < 0.05$, ** indicates $P < 0.01$.

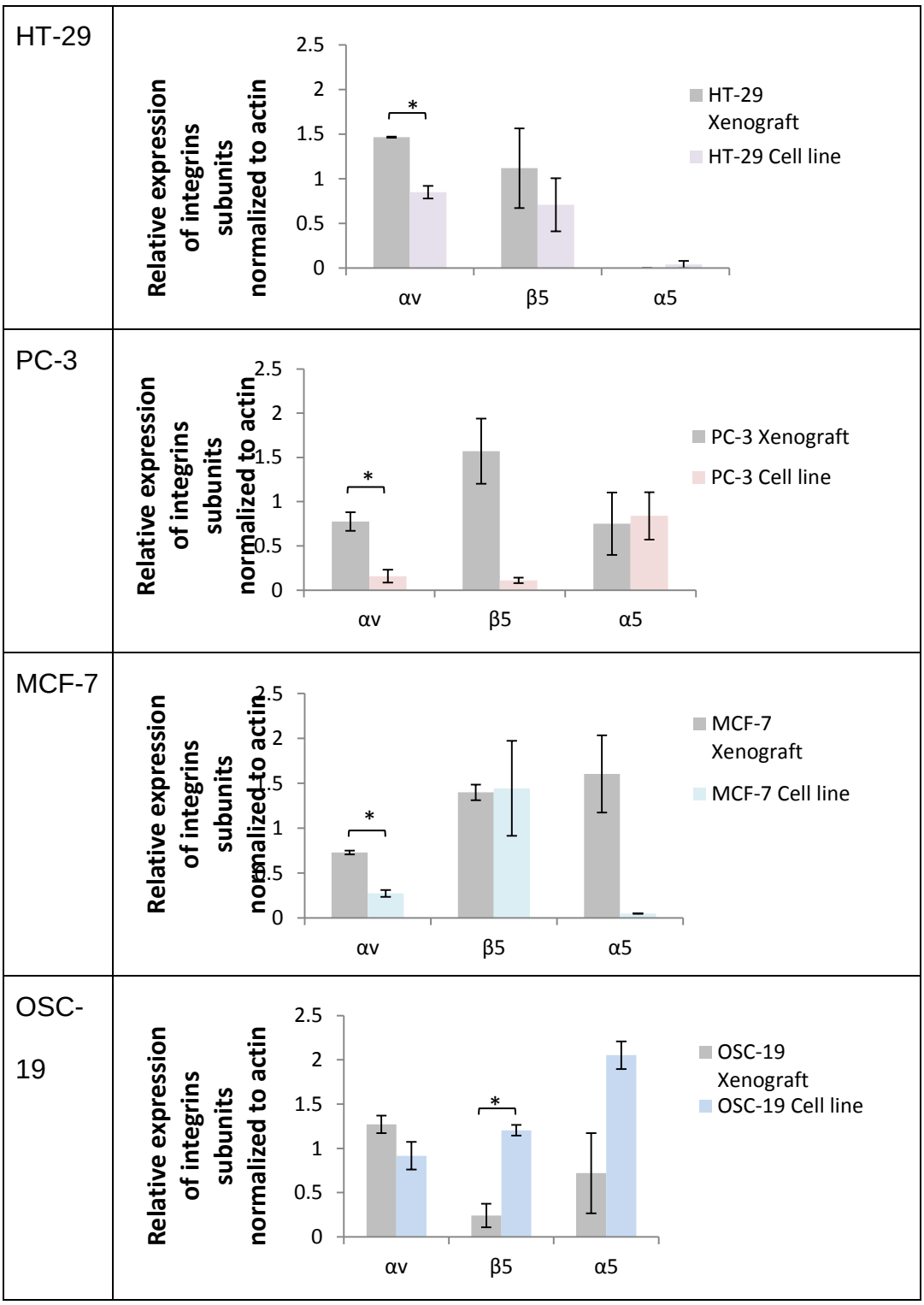


Figure 34 Comparison of the expression of αv, α5 and β5 integrin subunits in tumour cells by Western blot in tumour xenografts and in cell lines in vitro.

Results shown are the average of three independent experiments. * indicates $P < 0.05$.

MCF-7	Expression of integrin subunits in tumour xenografts	Expression of integrin subunits in cell lines *in vitro*	Expression of integrin subunits in tumour xenografts vs in cell lines *in vitro* (P value)
αv	++	+	0.01
β5	+++	+++	1
α5	+++	-	0.1
β3	++	n/a	
OSC-19			
αv	+++	++	0.1
β5	+	+++	0.03
α5	++	+++	0.1
β3	++	n/a	
PC-3			
αv	++	+/-	0.03
β5	+++	+	0.1
α5	++	++	0.2
β3	+++	n/a	
HT29			
αv	+++	++	0.04
β5	+++	++	0.4
α5	-	-	0.3
β3	+	n/a	

Table 18 Comparison of the expression of αv, α5 and β5 integrin subunits in tumour cells by Western blot in tumour xenografts and in cell lines in vitro.

3.5 Discussion

The integrin expression in clinical tumour samples described in Chapter 2 suggests head and neck and prostate cancer would be suitable clinical problems to address by multi-integrin targeting. Therefore, this chapter assessed the expression of αv, β5, and α5 subunits in head and neck (OSC-19) and prostate (PC-3 and DU145) cell lines by immunofluorescence and Western blot. The expression of αv, β5, and α5 subunits in other cell lines reported in the literature to have high or low expression of αv, β5 and α5 integrin subunits was also investigated: human breast carcinoma cells MCF-7; MCF-7ADR/NCRI ADR-RES cells as a drug-resistant model (Taherian et al., 2011, Nista et al., 1997); colorectal adenocarcinoma cells DLD-1 and HT-29 (Haier et al., 1999, Petersen et al., 2013); melanoma cells M14 and human umbilical vein endothelial cells HUVEC (Goodman et al., 2012) were evaluated by immunofluorescence and Western blot.

Furthermore, this chapter investigates the expression of αv, β3, β5, and α5 subunits in human tumour xenografts in order to identify *in vivo* models that can be used to test the efficacy of novel integrin antagonists as drug targets in cancer therapy.

3.5.1 Integrin expression in cell lines

Using immunofluorescence, the antibody recognizes the target protein in its native state, but with Western blotting, the antibody recognizes a denatured form of the protein caused by SDS/detergents, reducing agents and heat.

Furthermore, with immunofluorescence, some antibodies react with target and non-target proteins giving false-positive results. Therefore, Western blotting was used to determine the specificity and selectivity of the antibodies. It must be noted that, unfortunately, the expression of the integrins might be underestimated due to the overexposure of the western blots. The expression of the αv, β5, and α5 integrin subunits by immunofluorescence and western blot is summarised in Table 19.

3.5.1.1 The expression of αv and β5 integrin subunits

In this study, Q20 (anti-αv) and ab15459 (anti-β5) antibodies were used to evaluate the expression of αv and β5 integrin subunits on a panel of cell lines. With the breast cancer cells, the immunofluorescence results demonstrated that MCF-7 and MCF-7ADR cells express low levels of αv and high levels of the β5 integrin subunit on their surface. The western blot results confirmed the immunofluorescence results, but moderate expression of the β5 integrin subunit was observed in MCF-7 ADR. Meyer *et al.* detected that the αvβ5 integrin is expressed in many cell lines, such as ZR75-1, MDA-MB-231, MDA-MB-468, T47D, BT474, SKBR3, MCF-7, and BT20 and showed that αvβ5 integrin is the dominant vitronectin receptor in these cells (Meyer et al., 1998). Moreover, Taherian *et al*. found that normal cells express low levels of β3, β5, αvβ5 and αvβ6, whereas the cancer cell lines expressed high levels of β1, β5 and αvβ5. Interestingly, they found that only MDA-MB-435 expressed high levels of β3 and αvβ3 (Taherian et al., 2011) which is consistent with its possible origin from the M14 melanoma cell line. These studies may indicate that αvβ5 is the dominant

vitronectin receptor in cells of epithelial lineage. It has been shown that αvβ5 integrin is implicated in breast cancer progression as its expression regulates breast cancer motility (Zhang et al., 2002, Doerr and Jones, 1996), apoptosis (Lin et al., 2004),(Jandova et al., 2012) and metastasis (Bianchi et al., 2010a).

The expression of αv is well documented in human breast tissue. Pignatelli showed weak to moderate expression of αv subunit on non-neoplastic epithelium and in 13/19 invasive ductal carcinomas and 15/17 invasive lobular carcinomas (Pignatelli et al., 1992). Coene *et al*. found that 64% of invasive ductal adenocarcinoma tissues express high level of αvβ5 integrin (Coene et al., 1997). More recently, Goodman *et al*. evaluated the presence of αvβ5 and αvβ3 integrins on invasive ductal breast carcinoma tissues and they found that αvβ3 integrin was only present on vasculature, whereas αvβ5 integrin was restricted to interstices and vasculature (Goodman et al., 2012).

For the prostate cancer cell lines, the immunofluorescence and western blot results show αv integrin subunit expression to be very low and low in PC-3 and DU145 cell lines respectively. These results slightly differ from those of Zheng *et al.* and Goodman *et al*., who each found moderate expression of the αv integrin subunit in PC-3 cells using flow cytometry (Zheng et al., 2000), (Goodman et al., 2012). There are a number of possible reasons for the differences in integrin expression detected between studies, including different integrin detection methods, different specificity of antibody, different cell culture conditions, PC-3 cell heterogeneity, and the heterogeneity of cell subpopulations after long-term culture (Stachurska et al., 2012).

Furthermore, I found PC-3 and DU145 cells to express the β5 integrin subunit, with good correlation between immunofluorescence and the western blot results in this regard (Table 19). Goodman *et al*. and Bauer *et al*. reported little expression of β5 integrin subunits in PC-3 and DU145 cells (Goodman et al., 2012, Bauer et al., 2007). However, Bisanz *et al*. showed that αvβ1 and αvβ5 integrins mediate PC-3 cell adhesion to and migration on vitronectin. Knocking down αv integrin expression in PC-3 cells inhibited the growth of PC-3 tumours in bone due to enhanced tumour-cell apoptosis in bone (Bisanz et al., 2005).

With the colon cancer cell lines, the αv integrin subunit was moderately expressed and the β5 integrin subunit was moderately and highly expressed in HT-29 and DLD-1 cells respectively. Haier *et al*., also demonstrated low expression of αv and β5 integrin subunits in HT-29 using immunocytochemistry and western blot (Haier et al., 1999), whereas Goodman, found high expression of αv subunit in HT-29 cells by flow cytometry (Goodman et al., 2012). Studies showed that the expression of αvβ5 plays an important role in the adhesion of HT-29 within the hepatic microvasculature (Enns et al., 2005b). Yoshioka *et al*. found that αvβ5 requires hepatocyte-derived heregulin to mediate cell migration on VN (Yoshioka et al., 2010). αvβ5 has been shown to suppress the apoptosis of colon carcinoma cells induced by serum deprivation (O'Brien et al., 1996).

For the head and neck cancer OSC-19 cells, our results show that these cells over-express αv and β5 integrin subunits. Supporting these results are the immunofluorescence results conducted by Kawahara *et al*. (Kawahara et al.,

1995). In addition, it has been shown that αvβ5 integrin is highly expressed in larynx and tongue cancers, and its expression is associated with lymphatic metastasis and angiogenesis (Li et al., 2013, Kurokawa et al., 2008).

With regard to melanoma, I assessed the expression of αv and β5 integrin subunits in M14 cells using western blot only and the results showed that M14 cells moderately and highly expresses αv and β5 integrin subunits respectively. These results are slightly different from the flow cytometry results of Goodman *et al*. who found high and moderate expression of αv and β5 integrin subunits respectively (Goodman et al., 2012).

The immunofluorescence and western blot results show that HUVEC cells express high and moderate levels of the αv integrin subunit respectively. Both immunofluorescence and western blot results show that HUVEC cells express moderate levels of the β5 integrin subunit. These results are in agreement with what has been reported in the literature (Goodman et al., 2012, Dormond et al., 2001). Dormond *et al.* investigated the role of αvβ5 and αvβ3 integrins in the adhesion, spreading, and migration of HUVEC cells on vitronectin using monoclonal antibodies that block the function of αvβ3 (LM609) and αvβ5 (P1F6) and found that, while LM609 completely blocked HUVEC adhesion, P1F6 had no effect (Dormond et al., 2001). However, Hood *et al*. found that αvβ5 integrin uses FAK/Src signalling pathway to stimulate endothelial cell proliferation, differentiation and migration (Hood et al., 2003). Also αvβ5 protect the cells from intrinsic-mediated apoptosis by activating Raf and MEK1-dependent (Alavi et al., 2003).

3.5.1.2 The expression of the α5 integrin subunit

In this study, I used three antibodies to evaluate the α5 integrin subunit: ab93943, ab130968, and C-9 antibodies. The ab93943 antibody gave very clear membranous staining in immunocytochemistry, whereas western blot detected a single band with a molecular weight of roughly 20 kDa, which was smaller than the expected molecular weight of 115 kDa, and was expressed only in prostate cancer cell lines. This called into question the identity or specificity of the antibody supplied. The second anti-α5 (ab130968) antibody also gave us unsatisfactory results, detecting two bands, a faint one of the correct molecular weight, and a very strong one of very small molecular weight. A possible explanation for this is that the α5 subunit contains disulfide bonds between heavy and light chains, and due to the use of β-mercaptoethanol, the disulfide bonds were broken, producing a heavy chain (135 kDa) and a light chain (20 kDa). However, although I avoided reducing agents, anti-α5 (ab130968) failed to detect α5. Finally, the C-9 antibody gave good results, recognizing a protein with a molecular weight of roughly 150 kDa, and gave membranous and cytoplasmic staining in immunofluorescence.

α5 was not expressed by MCF-7 cells but was high frequency expressed by MCF-7ADR. Nista *et al*., who found that Adriamycin-resistant breast cancer cells express high levels of α5β1. They provided evidence of the presence of FN receptors α5β1 and α4β1 on the surface of adriamycin resistant ADR MCF-7 human mammary carcinoma cells but not on the parent adriamycin-sensitive

type (WT) by using cytofluorimetry and confirming the results by reverse transcription polymerase chain reaction (RT-PCR). From adhesion assay results, they found that the α5β1 receptor is responsible for ADR MCF7 cells adhering to FN which delivers a mitogenic signal to quiescent ADR MCF-7 cells and re-enters MCF-7 ADR into the S-phase and prevents apoptosis in serum free conditions (Nista et al., 1997). Moreover, Morozevich reported that MCF-7 DOX expresses much more α5β1 integrin compared to MCF-7 (Morozevich et al., 2006). Also, they showed that MCF-7 DOX cells have a stronger ability to migrate through matrigel (*in vitro* invasiveness). This is due to the over expression of the α5β1 integrin which has the ability to regulate the expression of metalloproteinases 2 (MMP-2) through signalling pathways including PI-3K, Akt and Erk protein kinases or by direct recruitment of MMP-2 to the cell surface (Morozevich et al., 2006). Therefore, the α5β1 integrin becomes a target for therapeutic intervention to counter the survival and invasiveness of tumour cells (Morozevich et al., 2006).

In addition, Ghiso *et al*. found that the α5β1 integrin function is associated with the urokinase plasminogen activator receptor (uPAR) and epidermal growth factor receptor (EGFR). In tumour cells uPAR interacts with and activates α5β1 integrin, and this leads to increased FAK and ERK activities which, in turn, promote cancer cell migration, invasion, proliferation and leads to metastasis (Aguirre Ghiso et al., 1999). Another study confirmed the importance of the uPAR/α5β1 integrin interaction in cancer recurrence using small molecules that interact with integrin binding sites on uPAR. These molecules inhibit ERK activity and tumour growth and block metastases (Chaurasia et al., 2009)

The expression of α5β1 integrin is well documented as immunohistochemistry analysis showed that breast cancer tissues express high level of α5β1 integrin (Koukoulis et al., 1991). Moreover, the analysis of gene expression array data from breast cancer patients showed high level of α5 integrin which is associated with decreased survival (Nam et al., 2010).

For prostate cancer, the α5 integrin subunit was over-expressed in DU145 cells and moderately expressed in PC-3 cells. These results are similar to flow cytometry results reported by Witkowski *et al*., who found that PC-3, DU145, and 431P cells expressed α5, α3, and α6 (Witkowski et al., 1993). Recently, Stachurska *et al*. demonstrated high levels of α5β1 on the surface of PC-3 *via* flow cytometry. Inhibition of α5β1 decreased the adhesion and spreading of tumour cells (Stachurska et al., 2012).

For colon cancer, the α5 integrin subunit was not expressed by HT-29 cells and moderately expressed by DLD-1 cells. Schreiner *et al*. reported that the HT-29 cell line does not express the α5β1 integrin (Shinohara et al., 1999). Moreover, Haier *et al*. found the α5 integrin subunit was absent in colon cancer cell lines regardless of metastatic ability (Haier et al., 1999). α5β1 integrin has been implicated in the inhibition of colon cancer cells growth *in vitro* by reducing DNA synthesis and cellular proliferation and decreases tumorigenicity *in vivo* (Varner et al., 1995).

For head and neck cancer, OSC-19 cells expressed high levels of the α5 integrin subunit in contrast to the results of Kawahara *et al*., who reported that OSC-19 cells lacked the α5 integrin subunit (Kawahara et al., 1995). Studies

have shown that the expression of the α5β1 integrin in head and neck squamous cell carcinoma is associated with tumour metastasis, poor prognosis, and resistance to therapy (Eke et al., 2012, Shinohara et al., 1999).

For melanoma, the western blot results indicated that M14 cells express high levels of the α5 integrin subunit. The over-expression of the α5 integrin subunit in M14 was also seen by Del Bufalo *et al*., who assessed the expression of integrins by flow cytometry (Del Bufalo et al., 1998). Our results show that HUVEC cells express the α5 integrin subunit on their surface. This is the first study to investigate the expression of the α5 integrin subunit in the HUVEC cell line using immunofluorescence and western blot techniques. Many studies confirm the role of the α5β1 integrin in angiogenesis, finding α5β1 antagonists such as SJ749 and ATN-161 to inhibit angiogenesis and thus reduce tumour growth *in vivo* (Kim et al., 2002, Stoeltzing et al., 2003).

αv				
Cell line	IF	WB	In literature	References
MCF-7	+	+	+++; FC	(Taherian et al., 2011)
MCF-7 ADR	+	+		
PC-3	+/-	+/-	++; FC +/-; FC	(Zheng et al., 2000) (Goodman et al., 2012) (Witkowski et al., 1993)
DU145	+	+	++; FC	(Goodman et al., 2012)
HT-29	+++	++	+; WB and IF	(Haier et al., 1999)
			+++; FC	(Goodman et al., 2012)
DLD-1	+++	++	+++; RT-PCR.	(Bauer et al., 2007)
HUVEC	+++	++	+++; FC	(Goodman et al., 2012, Dormond et al., 2001)
M14		++	+++; FC	(Goodman et al., 2012)
OSC-19	+++	++	+++; IF	(Kawahara et al., 1995)
β5				
Cell line	IF	WB	In literature	References
MCF-7	+++	+++	+++; FC	(Taherian et al., 2011)
MCF-7 ADR	+++	++		
PC-3	+++	+	+; FC	(Goodman et al., 2012)
DU145	+	+++	+; Fc	(Goodman et al., 2012)
HT-29	++	++	+; WB and IF ++; FC	(Haier et al., 1999) (Goodman et al., 2012)
DLD-1	+++	+++		
HUVEC	++	++	+++; FC +/-; FC	(Goodman et al., 2012) (Dormond et al., 2001)

M14		+++	++; FC	(Goodman et al., 2012)
OSC-19	+++	+++	+++; IF	(Kawahara et al., 1995)
α5				
Cell line	IF	WB	In literature	References
MCF-7	-	-	+; FC	(Morozevich et al., 2006)
MCF-7 ADR	+++	+++	+++; FC and RT-PCR	(Nista et al., 1997)
PC-3	++	++	+++; FC	(Stachurska et al., 2012) (Witkowski et al., 1993)
DU145	+++	+++	+++; WB, IF and FC	(Witkowski et al., 1993)
HT-29	-	-	-; WB and IF	(Haier et al., 1999)
			-; FC	(Varner et al., 1995)
DLD-1	++	+++	++; FC	(Petersen et al., 2013)
HUVEC	+++	+++		
M14		+++	+++: FC	(Del Bufalo et al., 1998)
OSC-19	+++	+++	-; IF	(Kawahara et al., 1995)

Table 19 Analysis of integrin (αv, α5 and β5) distribution on a panel of human cell lines obtained from immunofluorescence (IF) and Western blot (WB) results.

(−): no expression, (+/-) very low, (+): low expression, (++): moderate expression, (+++): high expression, blank: not tested. FC; Flow cytometry , WB; Western blot, IF; Immunofluorescence, RT-PCR; Reverse transcription polymerase chain reaction.

3.5.2 Protein expression on xenograft tissues

3.5.2.1 Immunohistochemistry on xenograft tissues

In this study, the distribution of αv, β3, α5, and β5 integrin subunits in human tumour xenograft tissue in mice was characterized using immunohistochemistry and Western blot to compare the expression of integrins in xenograft tissues with the same tumour model *in vitro* because it is known that tumour microenvironments affect the expression pattern (Koshida et al., 2004, Taylor et al., 2012). This also allowed selection of an *in vivo* model that can be used in the future to evaluate the efficacy of novel integrin antagonists. The expression of integrins has been extensively studied in fresh frozen tissues, yet very rarely in FFPE tissues due to the lack of monoclonal antibodies that specifically stain integrins in FFPE tissues, and the fact that FFPE processes such as fixation and heating may mask epitopes and make antibody-antigen binding difficult (Goodman et al., 2012).

In this study, FFPE xenograft tissues were used to evaluate the expression of αv, β3, α5, and β5 integrin subunits because histomorphology is not optimally maintained with frozen tissue making the evaluation of integrin distribution a challenge with frozen tissue. Unfortunately, the immunohistochemistry performed on the FFPE tissues was unsuccessful in this study, even when using different antigen retrieval methods. Consistent with this, the literature shows that detection of integrin in FFPE tissue is challenging. Goodman and his group produced rabbit monoclonal antibodies against the extracellular domains of αv integrins that would react with integrins within FFPE tissues, however

although these antibodies have been used in several studies (Boger et al., 2013, Fabricius et al., 2011, Goodman et al., 2012) they have not been made available commercially.

The expression of αv and β5 integrin subunits was evaluated *via* immunohistochemistry in fresh-frozen xenograft tissue using rabbit polyclonal antibodies Q20 (anti-αv) and ab15459 (anti-β5), each of which showed cytoplasmic immunolabelling in the xenograft tissues. Through the immunohistochemical analysis of the expression of αv and β5 integrin subunits, it was determined that colon, breast, head and neck, and prostate xenograft tissues expressed αv and β5 integrin subunits. The αv integrin subunit was highly expressed in the tumour cells of HT-29, OSC-19, and MCF-7 xenografts, and moderately expressed in the tumour and stromal cells of PC-3 xenografts. Furthermore, the β5 integrin subunit was strongly expressed in the stroma of OSC-19, and moderately expressed in the tumour cells MCF-7 and HT-29, as well as both the tumour and stromal cells of the PC-3 xenograft tissue. The immunohistochemical tumour and stroma results concerning αv and β3 integrin subunit expression within OSC-19, PC-3, HT-29 and MCF-7 xenografts are summarized in Table 20.

3.5.2.2 Western blot on xenograft tissues

The evaluation of the expression of α5 and β3 integrin subunits in human tumour xenograft tissue from mice by immunohistochemistry was difficult because non-specific binding was observed with the mouse antibodies B7 (anti-β3) and C-9 (anti-α5). Even using the M.O.M. kit, non-specific binding of these

antibodies could not be suppressed. Therefore, fresh frozen xenograft tissues were used in Western blotting to evaluate the expression of α5 and β3 integrin subunits and to confirm their expression. With Western blot, B7 (anti-β3) and C-9 (anti-α5) gave two bands: one band located at molecular weights consistent with α5 and β3 integrin subunits (150 kDa and 120 kDa, respectively), and another intense band between 70 and 55 kDa. In order to prove that non-specific binding was a property of the mouse antibodies rather than non-specificity for the human integrins, membranes were incubated with secondary antibodies only. Normal mouse tissue and human xenograft mouse tissue demonstrated non-specific binding due to the reaction of anti-mouse antibodies on mouse tissue. Excluding the non-specific band, the expression of integrin can be estimated by measuring bands consistent with the integrin molecular weight. The expression of αv, β3, α5, and β5 integrin subunits in Western blot were consistent with the immunohistochemistry results.

This study represents the first evaluation of the expression of αv, β3, α5, and β5 integrin subunits in OSC-19 head and neck cancer xenografted tissue. OSC-19 expressed a high level of the αv integrin subunit, a moderate level of both the α5 and β3 integrin subunits, and a relatively low level of the β5 integrin subunit. Recently, Terry *et al*. evaluated the expression of αv and β3 integrin subunits in fresh frozen head and neck tumour xenografts (FaDu, SCCNij3, and SCCNij202) using immunohistochemistry and they found that tumour cells within these three tumour xenografts did not express αvβ3 integrin (Terry et al., 2014).

With the PC-3 xenograft, β5 and β3 integrin subunits were strongly expressed, whereas α5 and αv integrin subunits were moderately expressed. Bisanz *et al*.

detected the expression of αv, β3, and β5 integrin subunits in PC-3 cells *via* flow cytometry *in vitro* and by immunohistochemistry and PCR *ex vivo.* They found that the PC-3 xenograft expressed moderate levels of the αv integrin subunit (Bisanz et al., 2005). Taylor found that integrin β3 mRNA was elevated in PC-3 xenografts compared with corresponding cultured cells (Taylor et al., 2012). Our results are different from Zhou *et al*., who demonstrated, *via* immunohistochemistry, that HT-29 and PC-3 xenograft tissues express low and very low levels of the αvβ3 integrin, respectively (Zhou et al., 2011).

In this study the expression of integrin subunits αv, β5, β3, and α5 in the HT-29 xenograft tissue to range from high to none. Our results concerning αvβ3 integrin expression are similar to Zhou *et al*.'s, yet slightly different from Goodman *et al*., who found no expression the αvβ3 integrin in HT-29 xenograft tissue (Zhou et al., 2011). Goodman *et al*. found a high expression of αvβ5 in HT-29 xenograft tissue (Goodman et al., 2012).

This study demonstrated that MCF-7 xenografts express high levels of the α5 and β5 integrin subunits and moderate levels of αv and β3 integrin subunits. In support of these results is a study by Goodman *et al*. who reported a high expression of the αvβ5 integrin in MDA-MB468 xenograft tissue (Goodman et al., 2012). The role of αvβ5 and α5β1 integrins in breast cancer was discussed earlier. αvβ3 has been shown to promote breast cancer invasion *in vitro* and *in vivo* by binding to matrix metalloproteinase MMP-2 (Deryugina et al., 2001). In *in vitro* studies, αvβ3 integrin was proved to mediate adhesion for breast cancer within the lung (Felding-Habermann et al., 2001). *In vivo*, over expression of this integrin increased breast cancer bone metastasis (Zhao et al., 2007). Integrin

distribution in a panel of xenografts obtained *via* immunohistochemistry and Western blot are summarized in Table 20. The expression of αv, β3, β5 and α5 integrin subunits was increased in most xenograft tissue compared with the corresponding cultured cells except the expression of β5 and α5 integrin subunits was elevated in OSC-19 cultured cells. The difference between the expression of αv integrin subunit in HT-29, PC-3 and MCF-7 xenograft tissue and corresponding cultured cells and β5 integrin subunit in OSC-19 cultured cells and corresponding xenograft tissue was statistically significant (P= 0.04, 0.03, 0.01 and 0.03 respectively).

The difference between the expression of integrins *in vitro* and *in vivo* is because the expression of integrins depend on stimuli produced by the microenvironment such as fibroblasts and immune cells including macrophages, their secreted factors, such as vascular endothelial growth factor, transforming growth factor β, and various chemokines (Alphonso and Alahari, 2009). Studies showed that when tumour cells proliferate, the local blood supply becomes inadequate, leading to a hypoxic, nutrient-deprived tumour environment. The local hypoxic condition activates hypoxia-inducible factor-1 (HIF-1), resulting in the release of vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF2) and tumour necrosis factor alpha (TNF-α) from proliferating tumour cells. These factors stimulate the expression of αvβ5, αvβ3 and α5β1 integrins (Friedlander et al., 1995, Brooks et al., 1994a, Kim et al., 2000b). Also studies showed that chemokines, which are present *in vivo*, but not added to standard cell cultures, can regulate the expression and activity of integrins (Dehghani et al., 2014).

	IHC				WB			
	αv in tumour cells	αv in stroma	β5 in tumour cells	β5 in stroma	αv	α5	β5	β3
MCF-7	+++	-	++	-	++	+++	+++	++
PC-3	++	++	++	++	++	++	+++	+++
HT-29	+++	-	++	-	+++	-	+++	+
OSC-9	+++	-	-	+++	+++	++	+	++
Normal					-	-	+/-	-

Table 20 Analysis of integrin (αv, α5 and β5) distribution on a panel of xenografts obtained from immunohistochemistry (IHC) and Western blot (WB) results.

(−): no expression, (-/+) very low expression, (+): low expression, (++): moderate expression, (+++): high expression, blank: not tested.

3.6 Conclusion

This study demonstrated: that all cell lines (MCF-7, MCF-7 ADR, PC-3, DU145, DLD-1, HT-29, HUVEC, OSC-19 and M14) express the αv, and β5 integrin subunits on their surfaces but with different levels of expression. The α5 integrin subunit is expressed by all cell lines except HT-29 and MCF-7. The expression of αv, α5, β5 and β3 integrin subunits was generally upregulated *in vivo* compared to cell lines *in vitro*, most likely due to the influence of the microenvironment on outside-in cell signalling. Based on the results obtained from these evaluations, six cell lines were selected as models to be used to further evaluate the potential of novel small molecule integrin antagonists to inhibit single or multiple integrins *in vitro*: DLD-1 and DU145 cells express multiple RGD-integrins so will be used as a model to investigate combined inhibition of these integrins, PC-3 and OSC-19 as models to investigate dual integrin antagonists, and MCF-7 and HT-29 as αvβ5-expressing models. MCF-7, OSC-19 and PC-3 xenografts could be used as *in vivo* model to evaluate the effect of multiple integrin antagonism and HT-29 as model for αvβ5 integrin antagonists.

4 CHAPTER 4: Evaluation of the effect of small molecule integrin antagonists on tumour cell migration

4.1 INTRODUCTION

The most frequent cause of death for patients with cancer is the metastasis of cancer cells from a primary tumour to a distant organ. The metastatic process consists of multiple steps, including the detachment of tumour cells from a primary tumor, invasion through connective tissue, intravasation into blood or lymph vessels, migration and extravasation, further tissue invasion, adhesion, proliferation, and survival in another site of the body.

In cell migration, cells move on 2D surfaces, such as a basal membrane or ECM fibres. Invasion is movement of cells through a 3D matrix and this process requires adhesion and proteolysis of the ECM components. Tumour cells can migrate without invasion, but cannot invade without migration (Kramer et al., 2013).

Tumour cells migrate as single cells or expand in sheets, files, or clusters (collective migration). Single cell migration can be subdivided into amoeboid and mesenchymal movement. Mesenchymal migration is characterized by the movement of elongated, spindle-like cell bodies involved in focal adhesion and cytoskeletal contractility (Thiery, 2002). Alternatively, in amoeboid migration, cells move as rounded ellipsoids without need of focal attachment to the

extracellular matrix (Fackler and Grosse, 2008). In collective migration, cells move through the extracellular matrix while preserving functional cell-cell junctions (Vaughan and Trinkaus, 1966). The expression of genes involved in cell motility such as integrins is altered in cancer cells, and this enables cancer cells to respond to signals in microenvironments that stimulate tumour invasion. Studies have shown that integrins play a crucial role in tumour cell metastasis by mediating outside-in and inside-out signaling, and cross-talk mechanisms (Huttenlocher and Horwitz, 2011, Hood and Cheresh, 2002). Certain integrins, such as αvβ3 and αvβ5, enhance tumor metastasis (Vogetseder et al., 2013, Bianchi et al., 2010a), while other integrins, such as the β1 integrin subunit, sometimes decrease tumour dissemination (Yao et al., 2007). α5β1 integrin has shown both positive (Stachurska et al., 2012, McKenzie et al., 2013, Mierke et al., 2011) and negative correlations with metastasis (Schirner et al., 1998, Seftor et al., 1998). Therefore, the appropriate evaluation of α5β1 integrin expression and its role in cancer is crucial before targeting this integrin.

A comprehensive overview on the role of integrin in cancer metastasis and the potential of targeting integrin to prevent cancer progression have been provided in many reviews (Suyin et al., 2013, Seguin et al., 2015, Ganguly et al., 2013, Goswami, 2013, Sutherland et al., 2012). A strong interest has therefore developed to greater understand and potentially target integrin-mediated migration in cancer progression. A summary of the features of *in vitro* assays that have been used to study cell migration and invasion is given in Figure 35.

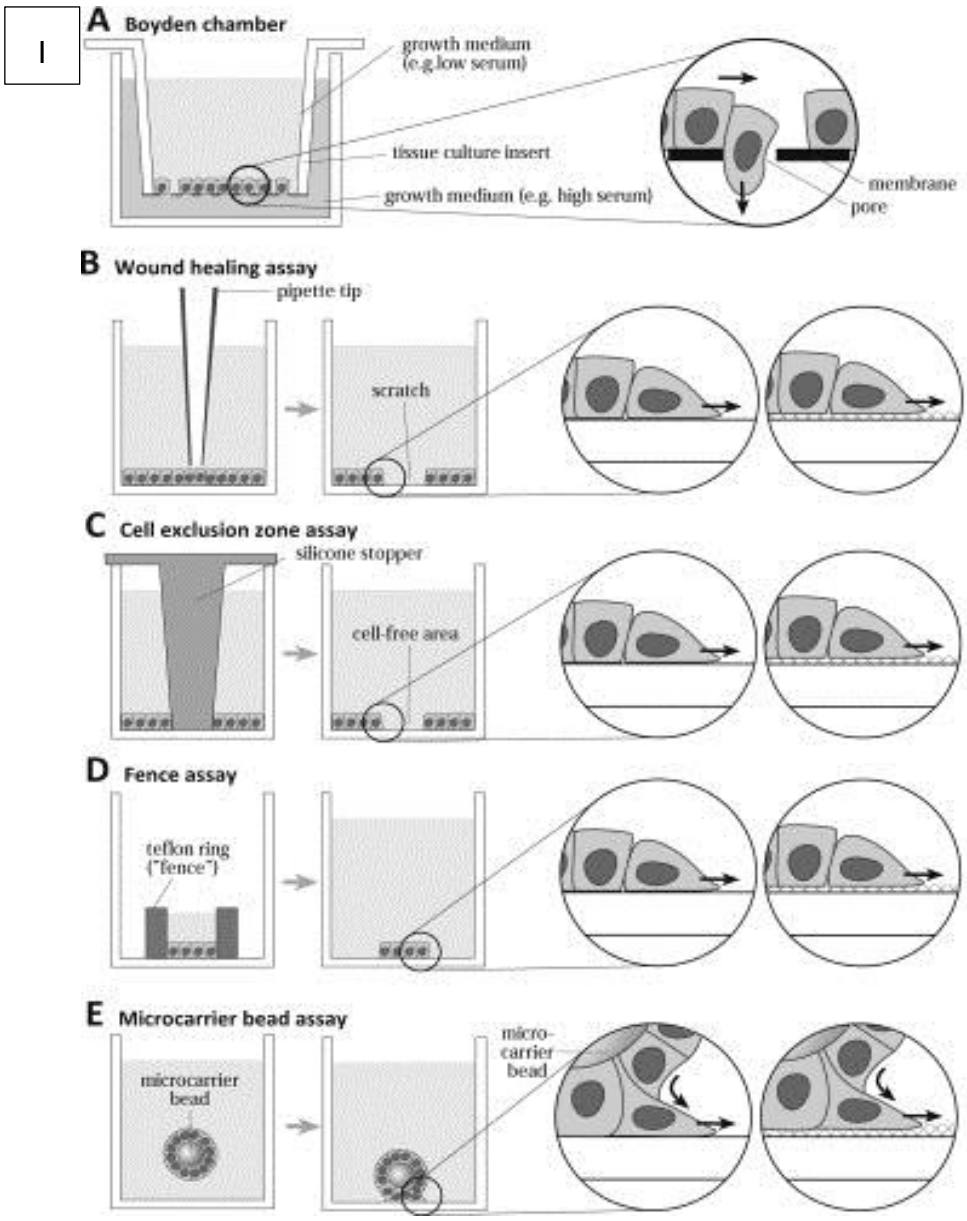


Figure 35. I Schemes of the most common migration assays.

(A) Transwell migration assay (Boyden chamber assay). Cells migrate through the pores within the membrane toward the chemoattractant in the well. (B) Wound healing assay. The cells migrate toward the cell-free area, which is created by scratching the cell monolayer with a P200 pipette tip. (C) Cell exclusion zone assay The cells migrate toward the cell-free area, which is created by utilising cell stoppers. (D) Fence assay. Cells are seeded inside a ring-shaped plastic device, which is removed after cell attachment. The cells migrate from the circular area to cell-free area. (E) Microcarrier bead assay. Cells are attached to the microcarrier beads surface and then beads coated with cells are placed onto cell culture dishes and incubated. Cells migrate to the plate surface and perform radial movement (Kramer et al., 2013).

II

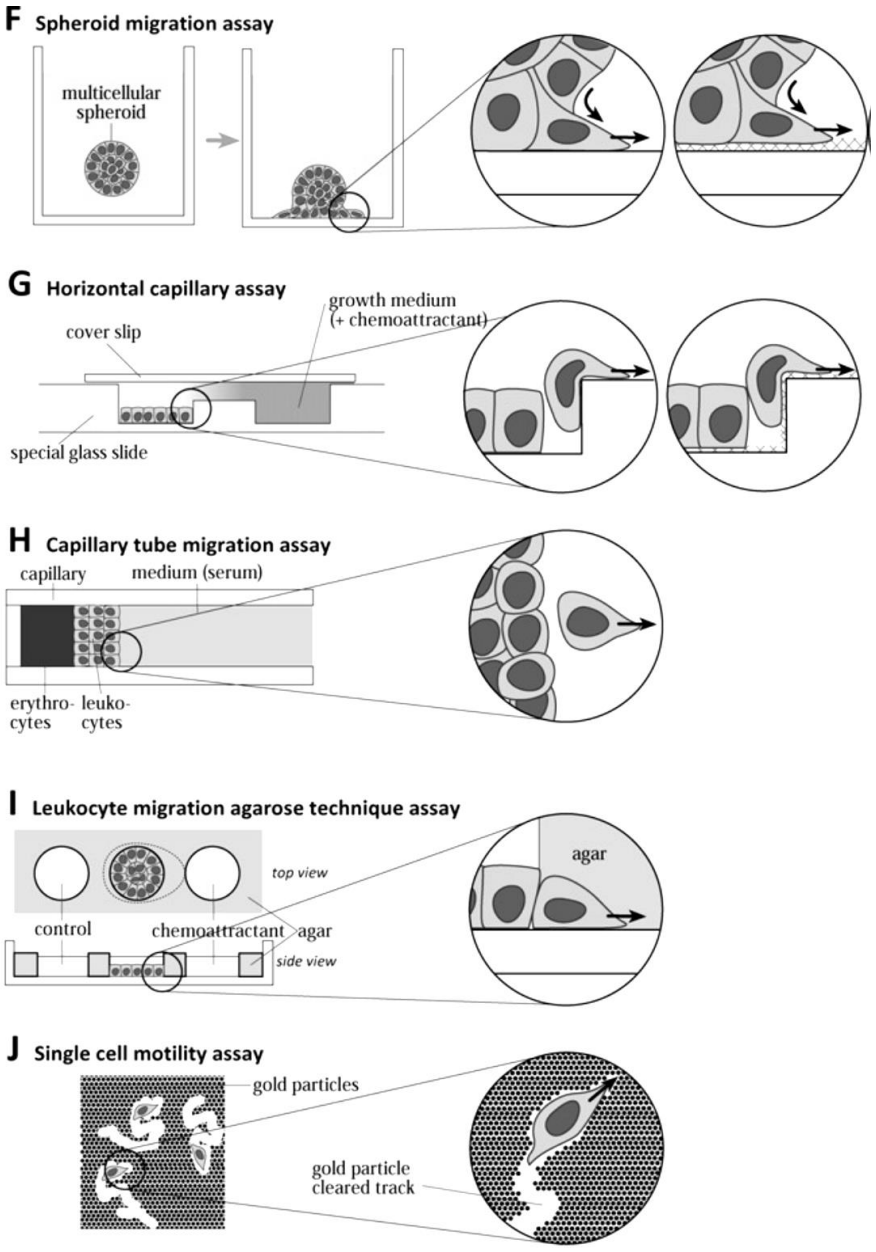


Figure 35. II Schemes of the most common migration assays (continued).

(F) Spheroid migration assay. Certain tumour cells have the ability to form multicellular spheroids. In this assay, the spheroid is placed in tissue culture dishes and the cells migrate concentrically outward. (G) Horizontal capillary assay. The cells migrate toward a chemoattractant within a capillary. (H) Capillary tube migration assay. Cells migrate into serum. (I) cells migration agarose technique assay. Cells migrate under the agarose layer toward the complete medium alone (control) or toward a chemoattractant. (J) Single cell motility assay. The plates are coated with colloidal gold particles and the cells are then added. During cell migration, the cells clear the gold particles, leaving bright tracks behind that can be measured.This method is now replaced by camera technology that films the cells in motion (Kramer et al., 2013).

In this study, a 2D wound-healing assay (scratch assay) was selected to evaluate the effect of integrin inhibition by neutralizing antibodies [LM609 (anti αvβ3 integrin), P1F6 (anti αvβ5 integrin), JBS5 (anti α5β1 integrin), and 10D5 (anti αvβ6 integrin)] and novel small molecule integrin antagonists on tumour cell migration.

The novel small molecule integrin antagonists synthesised in-house were designed to mimic the Arginine-Glycine-Aspartic acid (RGD) sequence of extracellular matrix proteins. They were designed by molecular modelling based on a novel scaffold between the Arginine and Aspartic acid mimetics as inspired by literature compounds.

While the scratch assay is simple, rapid, inexpensive, allows for easy readout, and analysis, it also has certain disadvantages, such as variable wound size and some cells still attach to the scratch edges after wounding and these cells reattach and move into the wound area. The scratch assay has been used extensively to evaluate the effect of integrin inhibitors on tumour cell migration. For example, Ray *et al*. demonstrated that blocking α5β1 or αvβ3/β5 integrins by small molecule antagonists inhibits U87MG cell migration (Ray et al., 2014). Janik *et al*. used scratch assays to evaluate the role of α3β1 and αvβ3 integrins in the migration of melanoma cells (Janik et al., 2010).

4.2 AIMS AND OBJECTIVES

The aim of the research described in this chapter was to evaluate the effect of novel small molecule integrin antagonists and the effect of antagonism of combinations of integrins on tumour cell migration *in vitro*. This was achieved by:

- Optimization of the migration assay using the cell line models characterised in Chapter 3.
- Determination which of four integrins (αvβ3, αvβ5, α5β1 and αvβ6) are primarily responsible for tumour cell migration by measuring cell migration under the influence of integrin-blocking antibodies LM609 (anti αvβ3 integrin), P1F6 (anti αvβ5 integrin), JBS5 (anti α5β1 integrin), and 10D5 (anti αvβ6 integrin).
- Evaluation of the cytotoxicity of the novel small molecule integrin antagonists by the MTT assay, in order to select a non-cytotoxic dose of each compound for each cell line model.
- Assessment of the effect of non-toxic doses of small molecule integrin antagonists on cell migration.

4.3 MATERIALS AND METHODS

4.3.1 Materials

The cell line models were maintained as described in Chapter 3. All cell culture reagents were obtained from Sigma-Aldrich unless indicated. 10 small molecule integrin antagonists that were synthesised by the Sheldrake group in the ICT were used. Compound stocks were prepared by dissolving compounds in 0.1% DMSO and then stored at −20 °C. Compound identification number and respective molecular weight is summarized in Table 21. The αIIbβ3 integrin antagonist GR144053 trihydrochloride (Tocris Bioscience) were stored at −20 °C. The antibodies and their applications are summarizedin Table 22.

Compound number	Molecular weight
ICT9019	503
ICT9023	475
ICT9024	503
ICT9026	475
ICT9055	542
ICT9072	474
ICT9073	516
ICT9085	500
ICT9087	542
ICT9088	468
ICT9094	557

Table 21 Compounds and their molecular weights

Applications	Primary antibody	Company	Secondary antibody	Company
Immunofluorescence	Rabbit polyclonal anti Ki-67 antibody	Millipore	Alexa Fluor 546 donkey anti rabbit IgG	Millipore
Western blot	C-20: goat polyclonal anti αIIb integrin subunit	Santa Cruz	Polyclonal rabbit anti goat linked to horseradish peroxidise	Dako
Western blot	ab15459: rabbit polyclonal anti β5.	Abcam	Polyclonal goat anti rabbit linked to horseradish peroxidise	Dako
Western blot	Q-20: rabbit polyclonal antibody directed to αv.	Santa Cruz	Polyclonal goat anti rabbit linked to horseradish peroxidise	Dako
Scratch assay	LM609: mouse monoclonal anti αvβ3 integrin	Millipore	n/a	
Scratch assay	P1F6: mouse monoclonal anti αvβ5 integrin	Millipore	n/a	
Scratch assay	JBS5: mouse monoclonal anti α5β1 integrin	Millipore	n/a	
Scratch assay	10D5: mouse monoclonal anti αvβ6 integrin	Millipore	n/a	

Table 22 Antibodies used in this chapter

4.3.2 Methods

4.3.2.1 MTT assay

1×10^4/ml of cells were seeded in 96-well plates in a final volume of 180 µl of medium and incubated for 24 hours at 37 °C in a 5% CO_2 humidified atmosphere. In addition to the test wells, one set included wells of culture medium containing no cells (blank), and one set included cells that were not exposed to compound (control). 20 µl of the compounds with different concentrations were added to all wells except the control and incubated for 72 hours at 37 °C in a 5% CO_2 humidified atmosphere. 200 µl of MTT solution was added to each and the plate was incubated for 4 hours at 37 °C in a 5% CO_2 humidified atmosphere. The liquid was then removed and 150 µl of DMSO was added and mixed in to dissolve the formazan crystals. The absorbance at 550 nm was measured using a Multiscan Plus spectrophotometer (Lab-systems Group, UK). The cell survival percentage was calculated as shown in the following equation. The IC_{50} values for each compound in each cell line were obtained from dose response curves.

$$\text{Cell survival \%} = \frac{\text{The absorbance of the test wells} - \text{The absorbance of blank}}{\text{The absorbance of control} - \text{The absorbance of blank}} \times 100$$

4.3.2.2 Scratch assay

The desired cell concentrations in 350 µl of RPMI 1640 medium were seeded into a 24-well plate and set overnight at 37 °C in a 5% CO_2 humidified

atmosphere to attach, spread, and form a confluent monolayer. A sterile P200 pipette tip was then used to scratch the confluent monolayer to form a cell-free zone (approximately 650 μm in width) into which the cells at the edges of the wound could migrate. The old medium was gently removed, and the cells were washed twice with PBS. After scratching the monolayer, one well was incubated with medium (control) and the other wells were incubated with different concentrations of antibody or antagonist The width of the initial scratch (time zero) was measured at three points throughout the scratch area in each well using a calibrated graticule fixed to a phase-contrast microscope (Olympus, model CK2) within a 10x objective lens. The cells were then incubated at 37 °C in a 5% CO_2 humidified atmosphere until the wound in the control well was 90% healed. The cells were then washed with PBS and left to dry. Following this, the cells were fixed with 70% ethanol for one minute, washed twice with PBS, and then placed in Harris's haematoxylin solution for one minute. The cells were then washed in tap water for one minute and left to dry at room temperature. The stained cells were assessed by inverted microscope and images were taken by camera (Nikon camera, model: C-D 55230, Japan) connected to an inverted microscope (Nikon model SMZ1500, Japan) at three points throughout the scratch area. The degree of wound closure or cell migration expressed as area occupied by cells (free area before migration minus free area after migration) was determined using ImageJ analysis to measure cell migration percentage within the captured images. Cell migration percentage was calculated in two ways: for cells that migrated as single cells:

$$\text{Cell migration percentage} = \left[\frac{\left[\frac{\text{Migrated cell count} \times \text{width at initial scratch}}{100} \right]}{\text{Control}} \right] \times 100$$

And for cells that migrated as a group:

$$\text{Cell migration percentage} = \frac{\left[\frac{\text{Free area before migration-free area after migration}}{\text{Free area before migration}} \right] \times 100}{\text{Control}}$$

Alternatively, images of the initial scratch (time zero) and the time point at which the wound in the control was 90% healed were taken at three points along the scratch field using a lumascope (Etaluma, model 488, USA). The degree of wound closure or cell migration expressed as area occupied by cells (free area before migration minus free area after migration) was determined using ImageJ analysis to measure cell migration percentage within the captured images. The cell migration percentage was then calculated as in the previous equation.

Statistical analysis of the difference from control was determined using a student's t-test. If the calculated P-value was below 0.05 or 0.01, the results were considered statistically significant or highly significant, respectively.

4.3.2.2.1 Distinguishing cell proliferation from cell migration in the scratch-wound assay

4.3.2.2.1.1 Evaluating the effect of different serum concentrations on cell migration

Cells were seeded in 24-well plates and incubated overnight to obtain a confluent cell monolayer. The scratch assay was carried out as described in section 4.3.2.2. After making the scratch, the cells were incubated with complete medium containing two different concentrations of fetal bovine serum (3 and 10%) at 37 °C in a 5% CO_2 humidified atmosphere until the wound in the control well was 90% healed.

4.3.2.2.1.2 Evaluating the proliferation marker Ki-67 by immunofluorescence

The desired cell concentrations were seeded on autoclaved coverslips in six-well plates and incubated overnight at 37 °C in a 5% CO_2 humidified atmosphere to attach, spread, and form a confluent monolayer. The monolayer was scratched and left to heal for the appropriate time for each cell line. In each experiment, two cover slips were used, one for the initial scratch (time zero), and the other for the last time point of wound healing. The immunofluorescence technique was carried out as described in Chapter 3. To evaluate cell proliferation, cells were fixed with 4% PFA and blocked with 5% BSA for one hour at room temperature. These cells were incubated with anti-Ki-67 antibody, 1:50 at 4 °C overnight, and then with the secondary antibody (Alexa Fluor 546 donkey anti rabbit IgG) 1:200 for one hour at room temperature.

4.3.2.2.2 **Evaluation of integrin expression by Western blot**

The scratch assay was set up as previously described but when the old medium was removed following the scratch, the cells were then washed twice with PBS and then cells in three wells were incubated with complete medium and other cells in three wells were incubated with 2.5 µg/ml of the JBS5 antibody (anti α5β1 integrin). The cells were incubated for 18 hours at 37 °C in a 5% CO_2 humidified atmosphere. The following day, the cells were washed twice with PBS prior to the addition of trypsin-EDTA, and the mixture was incubated for three minutes at 37 °C until the cells were completely detached. 3 ml of complete media was then added, and the cell suspension was centrifuged at room temperature for five minutes at a speed of 1000 RPM. The supernatant was then discarded and the cell pellets were re-suspended in 1 ml of PBS. The cell suspension was centrifuged again as per the prior step, the supernatant was discarded, and the cell pellets were stored at −20 °C. The Western blot procedure was carried out as described in Chapter 3.

4.4 RESULTS

4.4.1 Wound assay

4.4.1.1 Optimization of the scratch assay

In order to understand the wound healing process and study cell-cell and cell-extracellular matrix (ECM) interactions that mediate this response, the 2D scratch assay was used.

MCF-7 and HT-29 cell lines (models for high αvβ5 integrin expression and negative for α5β1 integrin) were found to be unsuitable for use in the scratch assay because MCF-7 cells did not form a monolayer and HT-29 cells did not migrate on the plastic surfaces investigated.

8.5×10^5 cells/ml was found to be the optimum cell seeding concentration for OSC-19, DLD-1, DU145, PC-3, M14 and HT-29 cell lines to form a monolayer after overnight incubation. After formation of the scratch wound, the OSC-19 cell line needed 18 hours to reach 90% healing, the DU145, PC-3 and M14 cell lines needed 24 hours and the DLD-1 cell line needed 41 hours. The HT-29 scratch did not heal even after 72 hours (Figure 36).

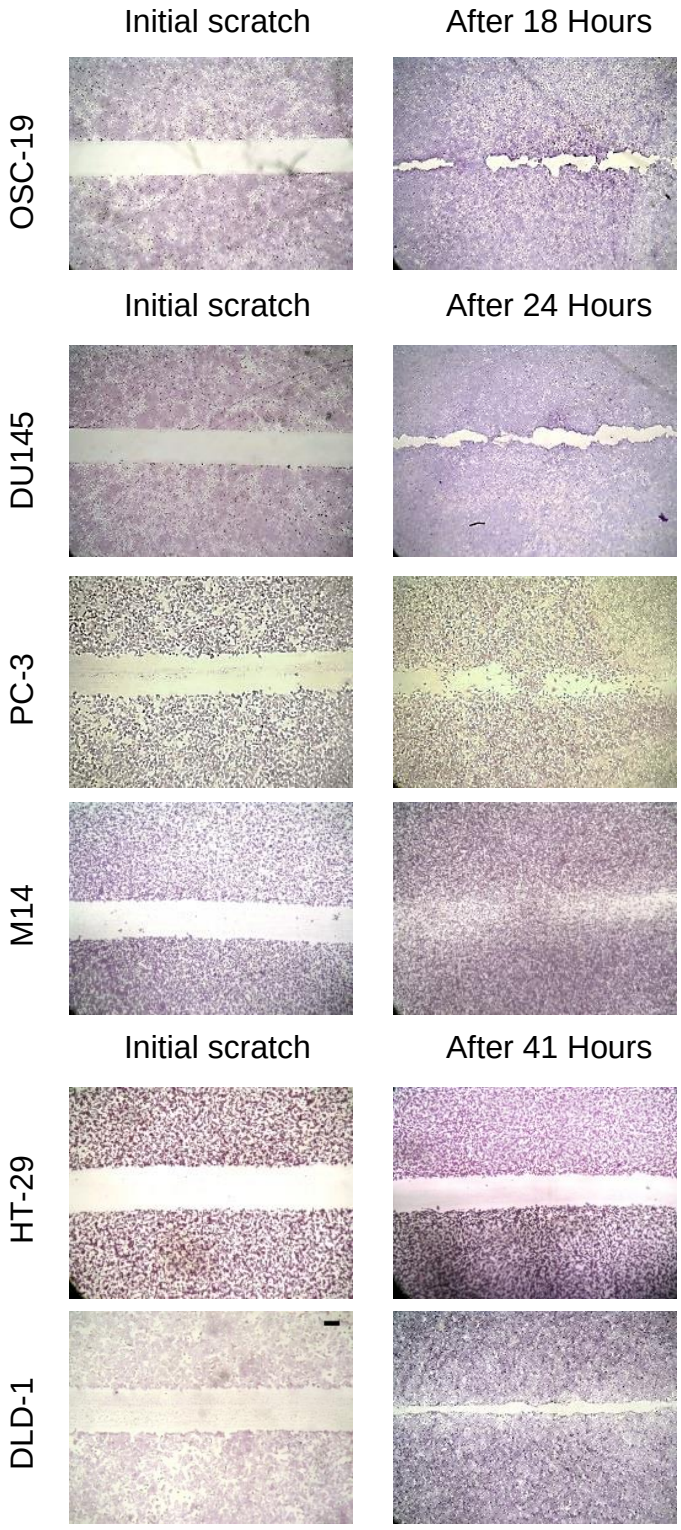


Figure 36 Evaluating the time required for wound closure in tumour cell line models.

The scratched monolayers were incubated with complete medium until the wound in the control was 90% healed. Images of cells stained with Harris's Haematoxylin solution. Scale bar = 200 µm.

4.4.1.1.1 Confirmation that the scratch-wound assay measures cell migration

In long term wound healing, it is difficult to differentiate between cell proliferation and migration. I used two methods to determine whether wound closure in the scratch assay was by migration or proliferation into the wound.

4.4.1.1.1.1 Evaluation of the proliferation marker Ki-67 and cell morphology by immunofluorescence

Immunostaining was carried out on scratched cells to detect Ki-67, which is a nuclear protein expressed by proliferating cells in all phases of the active cell cycle. The absence of Ki-67 in cells at the scratch edges confirmed that cells moving into the wound area were not proliferating and hence supported migration as the method of wound healing. The Ki-67 expression was higher at the area immediately behind the scratch edges in DLD-1 and DU145 cells. However, it is the cells at the scratch edge that are important because they are most migratory and the rate of their migration reflects the overall rate of wound closure of the migrating monolayer of cells (Rodriguez et al., 2005) (Figure 37).

4.4.1.1.1.2 Evaluation of the effect of serum concentration on cell migration

It is known that serum contains growth factors, cytokines and soluble ligands that may affect the rate of cell migration. Therefore, the effect of different serum concentrations on the migration rate was evaluated. In this experiment, OSC-19, DU145 and DLD-1 cell migration was monitored in a medium containing two different concentrations of FBS (low serum of 3% and the standard 10%). No

significant differences in migration promoted by the two concentrations were observed (P > 0.05) (Figure 38).

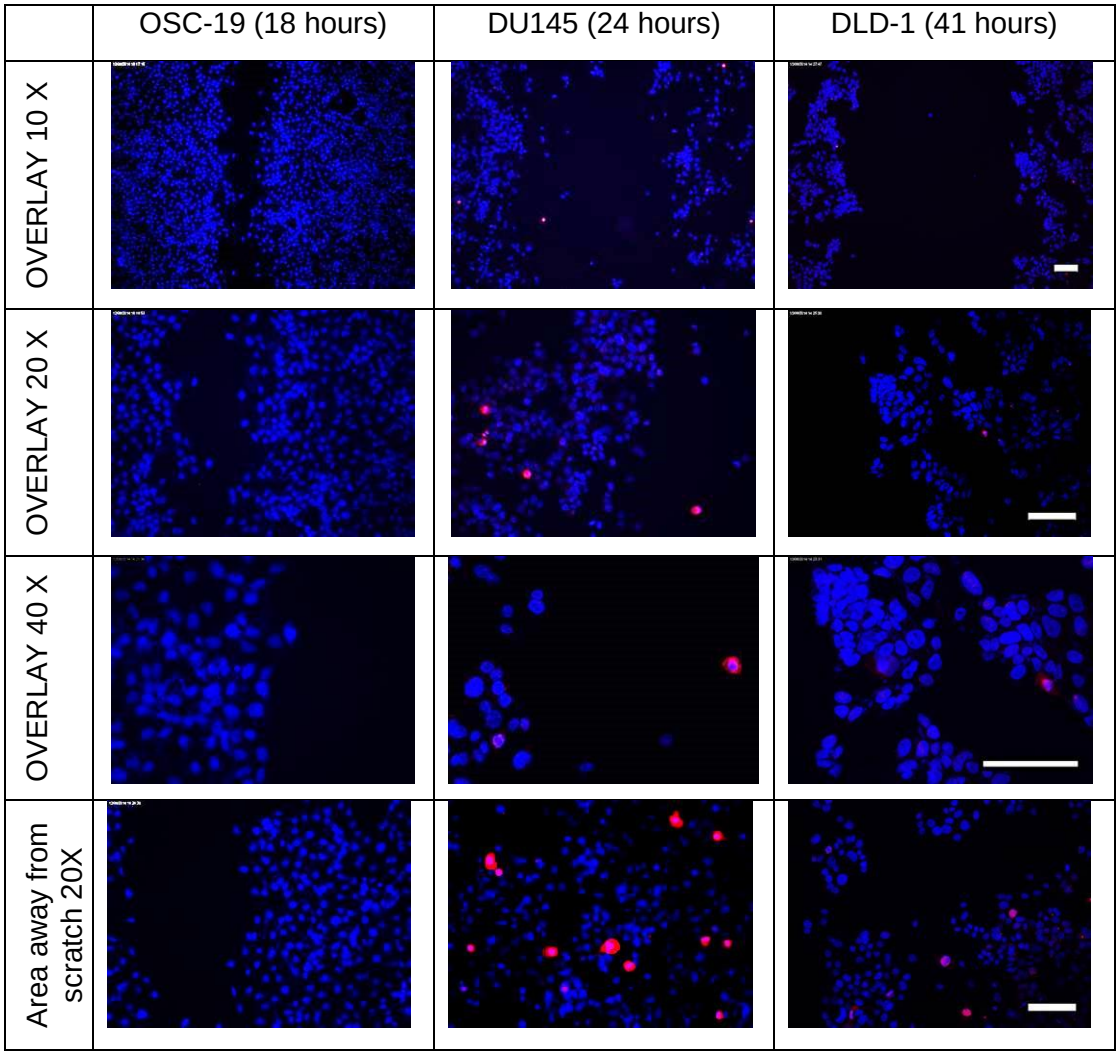


Figure 37 Immunofluorescence with anti-Ki-67 antibody.

OSC-19, DU145 and DLD-1 cells were scratched and incubated at 37 °C in 5% CO_2 humidified atmosphere for 18, 24 and 41 hours respectively. The cells were fixed with 4% PFA, and blocked with 5% BSA. Cells were incubated with Ki-67, 1:50 at 4 °C overnight. The expression of Ki-67 (red) was absent in OSC-19 and low on DU145 and DLD-1 cells located at the scratch edges. The cells located at area behind the scratch express moderate level of Ki-67. Scale bar = 100 µm.

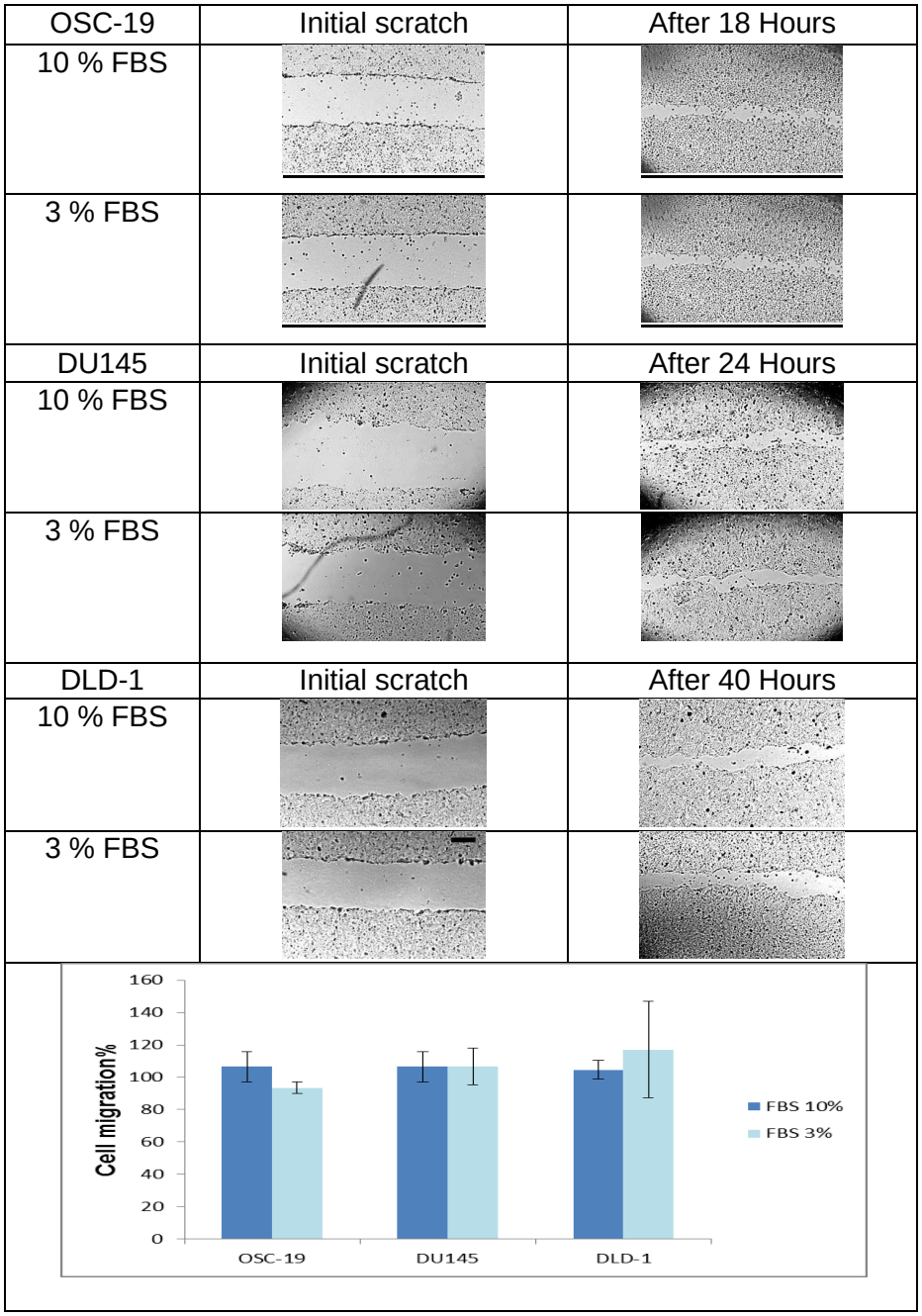


Figure 38 Effect of fetal bovine serum (3 and 10 %) on the wound assay

Scratched cells were incubated with two different concentrations (3 and 10 %) of fetal bovine serum (FBS). Images of OSC-19, DU145 and DLD-1 cells were taken by Lumascope after 18, 24 and 41 hours respectively. Scale bar = 200 µm. Results shown are average ± SD of three independent experiments. No significant difference between migration rates was observed ($P > 0.05$).

4.4.1.1.2 **Determination of which integrins control migration in OSC-19 cells**

To address which integrin is primarily responsible for OSC-19 cell migration, three integrin-blocking antibodies were used: LM609 (anti αvβ3 integrin), P1F6 (anti αvβ5 integrin) and JBS5 (anti α5β1 integrin). Based on the literature, the non-cytotoxic concentrations of these antibodies were used without first evaluating the cytotoxicity of these antibodies on this cell line.

OSC-19 cell migration was 25% and 30% inhibited by integrin receptor blocking with LM609 (anti-αvβ3) 2.5 μg/ml and PIF6 (anti-αvβ5) 2.5 μg/ml respectively, whereas JBS5 (anti-α5β1) 2.5 μg/ml gave an agonist effect, increasing migration by 80% (Figure 39).

To investigate the reason for this agonist effect, the impact of treatment with JBS5 on the expression of β3 and β5 integrins was determined. I found that blocking α5β1 integrin increased the expression of β3 and induced ectopic expression of αIIb. The expression of β3 and αIIb integrin subunits in stimulated cells was 42 and 13 fold higher than control respectively (Figure 40). The expression of αvβ5 integrin was not changed.

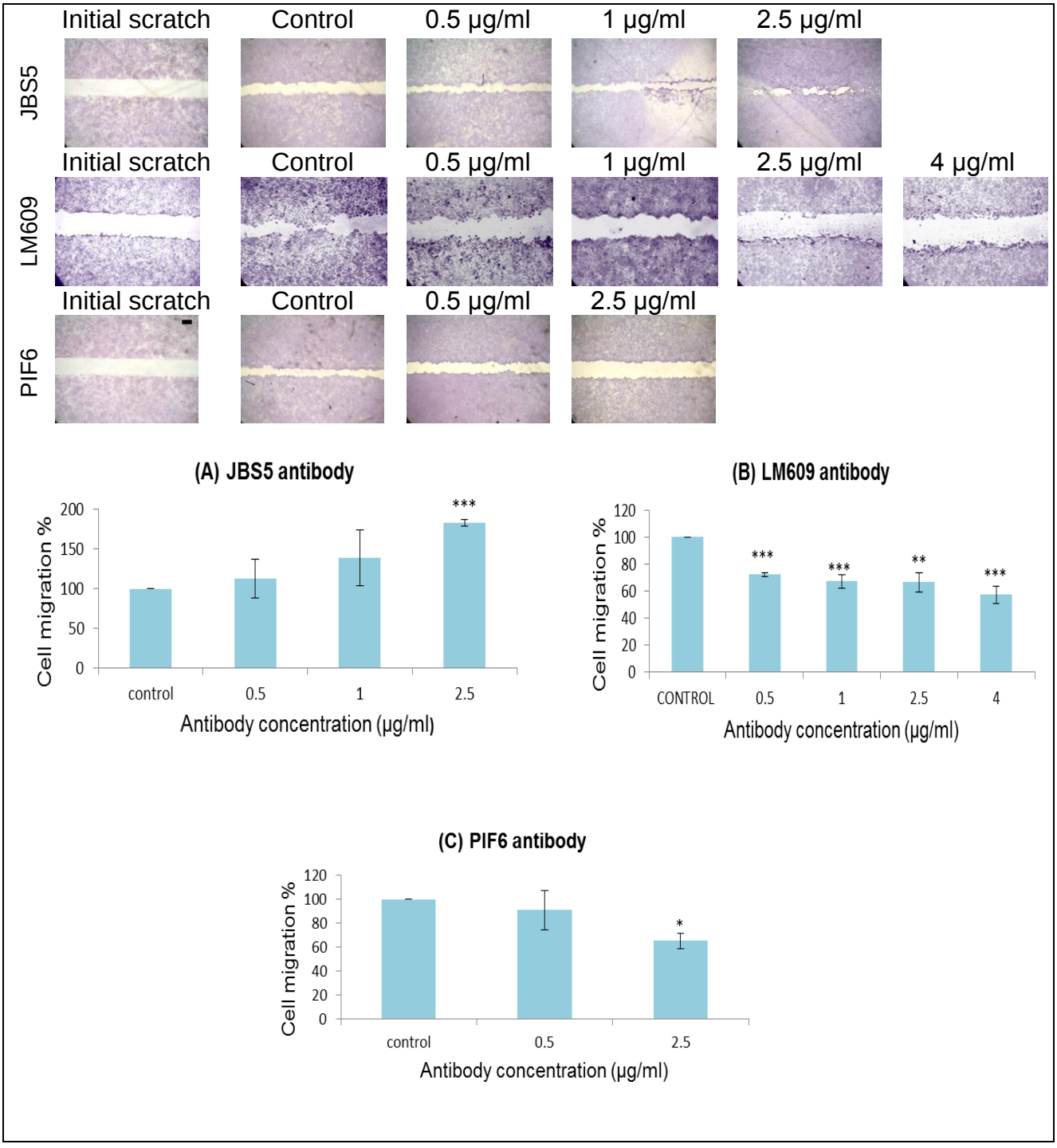


Figure 39 The effects of integrin-receptor blocking with neutralizing antibodies on OSC-19 cell migration using the scratch assay.

OSC-19 cells were treated with different antibody concentration for 18 hours. The cell migration was inhibited by LM609 and PIF6 antibodies but JBS5 antibody increased OSC-19 cell migration. Results shown are representative of three independent experiments. Bar length = 250 µm. Results shown are average ± SD of three independent experiments. * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

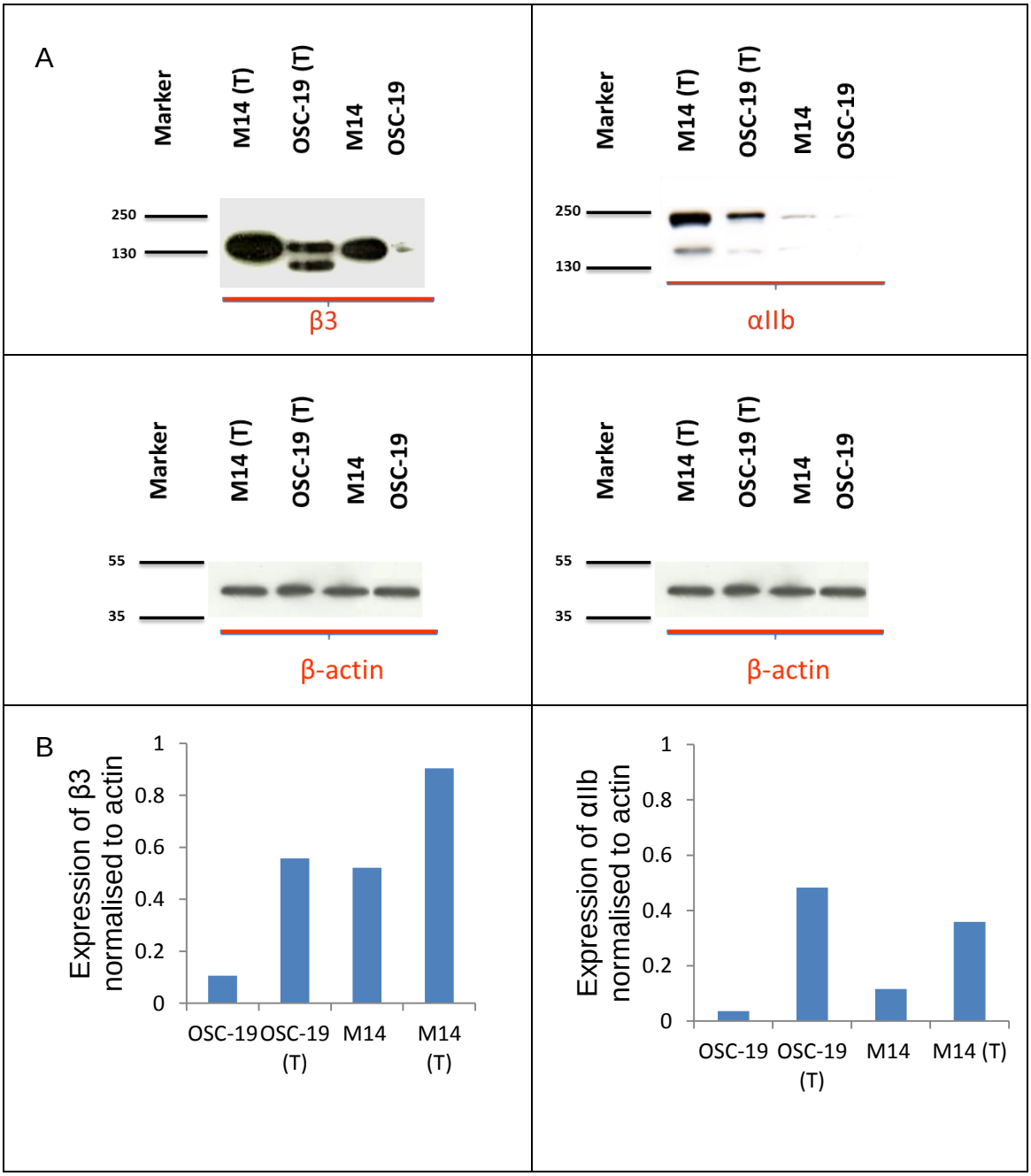


Figure 40 Alteration in integrin expression with JBS5 treatment.

OSC-19 and M14 cells were stimulated with JBS5 (anti-α5β1) 2.5 µg/ml for 18 hours prior to Western blotting for integrin expression. (A) Lanes 1 + 2 antibody treated cells (T), Lanes 3 + 4 untreated controls. Membranes were blotted with anti β3 (B-7), anti αIIb (C-20) and anti β-actin antibodies, which recognize proteins with band size 125, 136 and 42 kDa respectively. (B) Integrin expression levels normalised to β-actin calculated from three independent experiments.

4.4.1.1.3 **The effect of combination treatment on OSC-19 cell migration**

These findings demonstrate that blocking of integrin α5β1 increases β3 integrins expression, and notably induces αIIb expression, which is likely to result in αIIbβ3 surface expression and increased cell migration *via* αIIbβ3 and αvβ3. Therefore, the small molecule αIIbβ3 integrin antagonist GR144053 was used to evaluate the effect of blocking αIIbβ3 on OSC-19 cell migration in JBS5 treated cells. I found that GR144053 gave dose-dependent inhibition of cell migration; 200 μM inhibited the cell migration by 30% (Figure 41).

I then evaluated the effect of a multi-integrin inhibitor combination on OSC-19 cell migration using the scratch assay; a significant reduction in migration (50%) was observed using a multi-integrin inhibitor combination that blocks α5β1, αvβ3, αvβ5 and αIIbβ3 integrins (Figure 42).

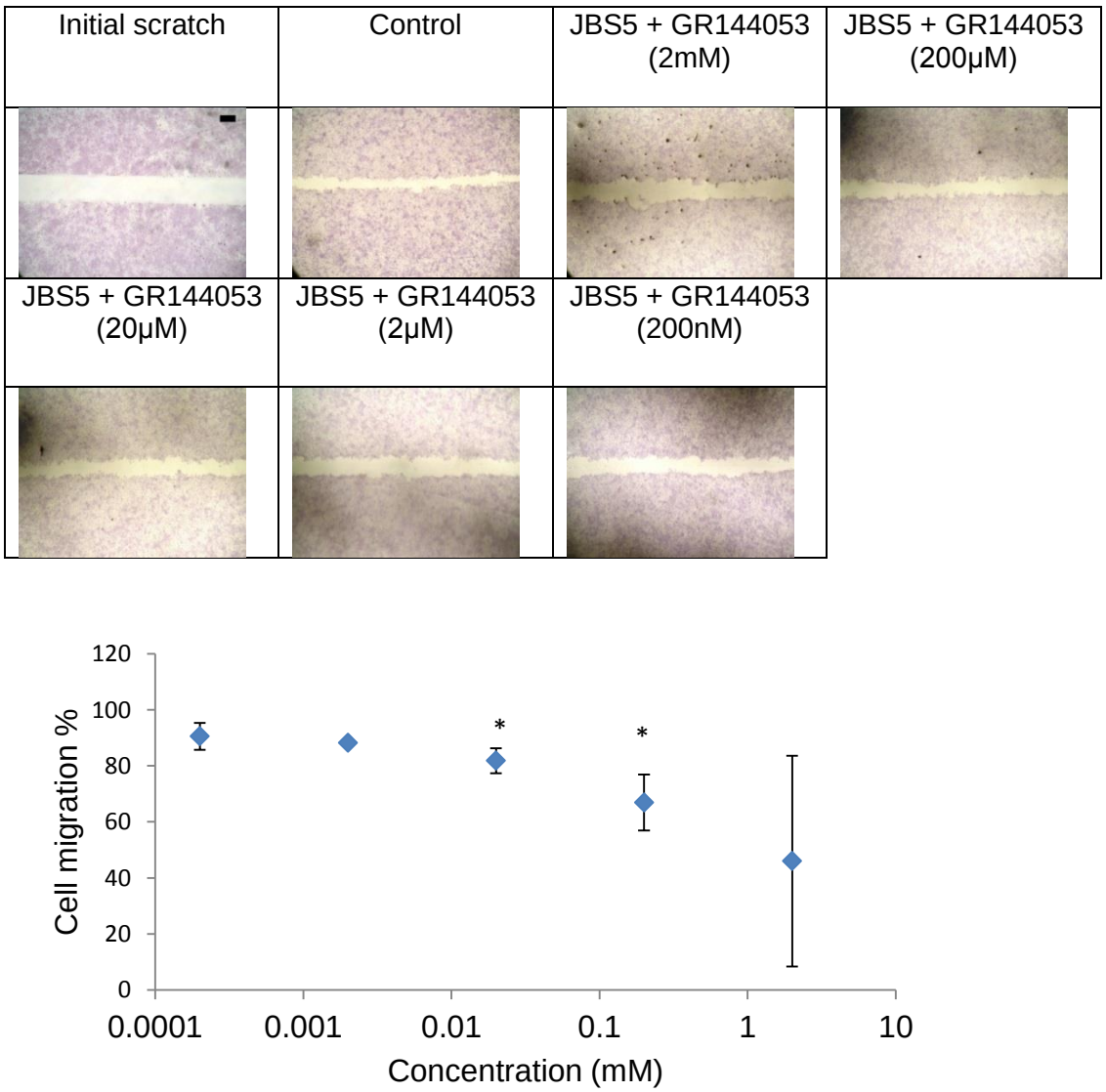


Figure 41 A dose-response curve

OSC-19 treated with 2.5 μg/ml of JBS5 (anti-α5β1) antibody with different concentrations of αIIbβ3 antagonist GR144035. There is a significant reduction in cell migration with 200 μM GR144035. Scale bar = 250 μm. Results in the graph calculated from three independent experiments. * indicated $P < 0.05$.

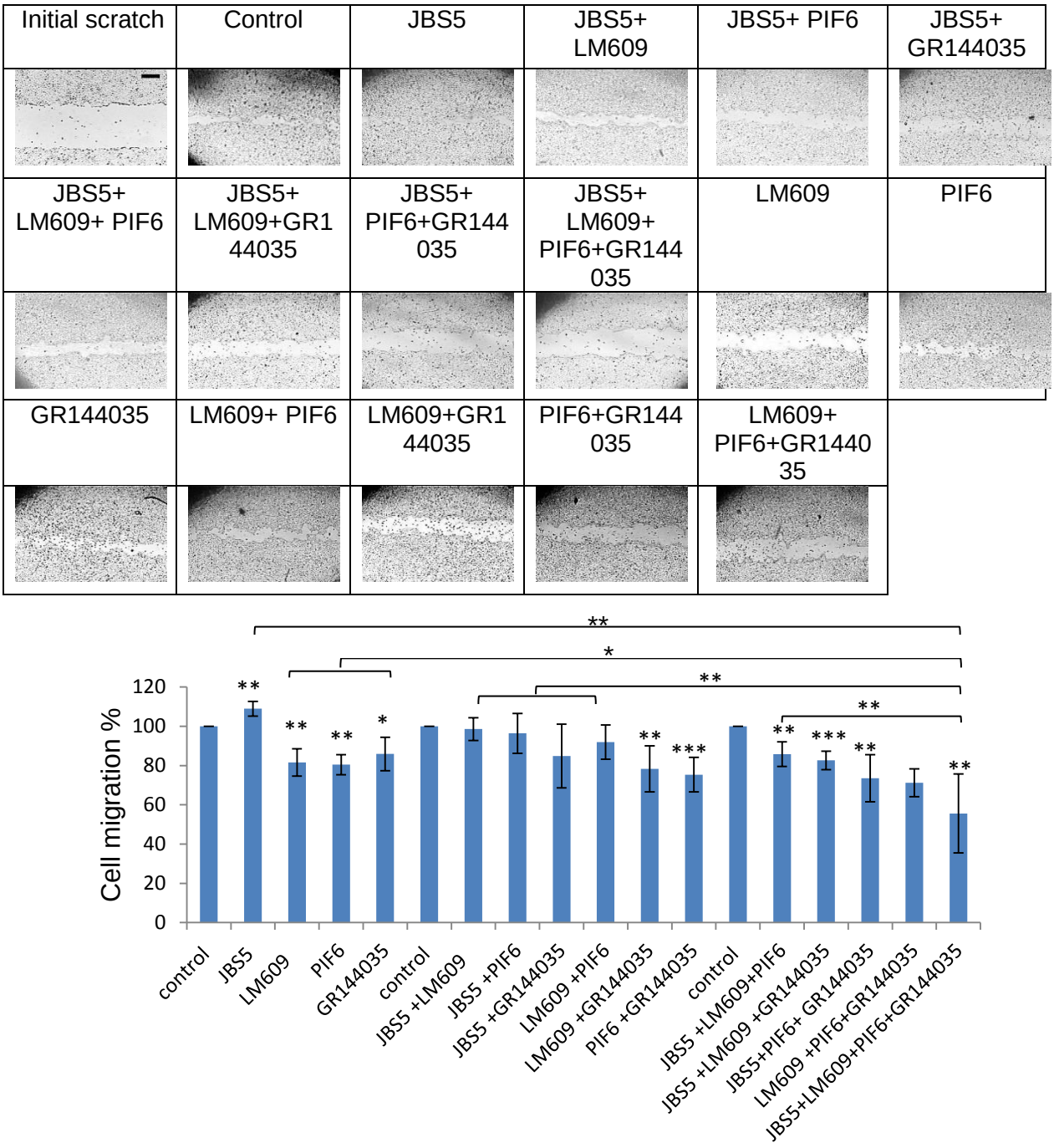


Figure 42 Effects of multi-integrin inhibitor combinations on OSC-19 cell migration.

OSC-19 treated with one or more antagonists: 0.5 µg/ml of JBS5 (anti-α5β1), 2.5 µg/ml of LM609 (anti-αvβ3), 2.5 µg/ml of PIF6 (anti-αvβ5), 200 µM GR144035 (anti-αIIbβ3) for 18 hours. Scale bar = 200 µm. Results in the graph calculated from three independent experiments. The asterisk * indicates $P < 0.05$, ** indicates $P < 0.01$ and*** indicates $P < 0.001$.

4.4.1.1.4 **Determination of which integrins control migration in DLD-1 cells**

To address which integrin is primarily responsible for DLD-1 cell migration, four integrin-blocking antibodies were used LM609 (anti αvβ3 integrin), P1F6 (anti αvβ5 integrin), JBS5 (anti α5β1 integrin) and 10D5 (anti αvβ6 integrin). The results suggest that JBS5 antibody had negligible effect on DLD-1 cell migration. DLD-1 showed a dose dependent response to PIF6 antibody (Figure 43 and Figure 44).

Antagonists of αvβ3 and αvβ6 showed dose-dependent effects on DLD-1 migration but the patterns were the inverse of one another. LM609 at 5 and 2.5 µg/ml inhibited DLD-1 cell migration by 45%, but the lowest concentration, 1 µg/ml, had little effect on cell migration. 5 and 2.5 µg/ml concentrations of 10D5 antibody had a negligible effect on the migration of cells. However, a significant reduction was observed using the lowest concentration of 10D5 antibody (1 µg/ml) (Figure 43 and Figure 44).

Moreover, I evaluated the effect of a multi-integrin inhibitor combination on DLD-1 cell migration using the scratch assay and found that blocking both αvβ3 and αvβ5 integrins simultaneously caused a significant decrease in cell migration. However, no further reduction was observed when anti-α5β1 was added to the combination (Figure 45).

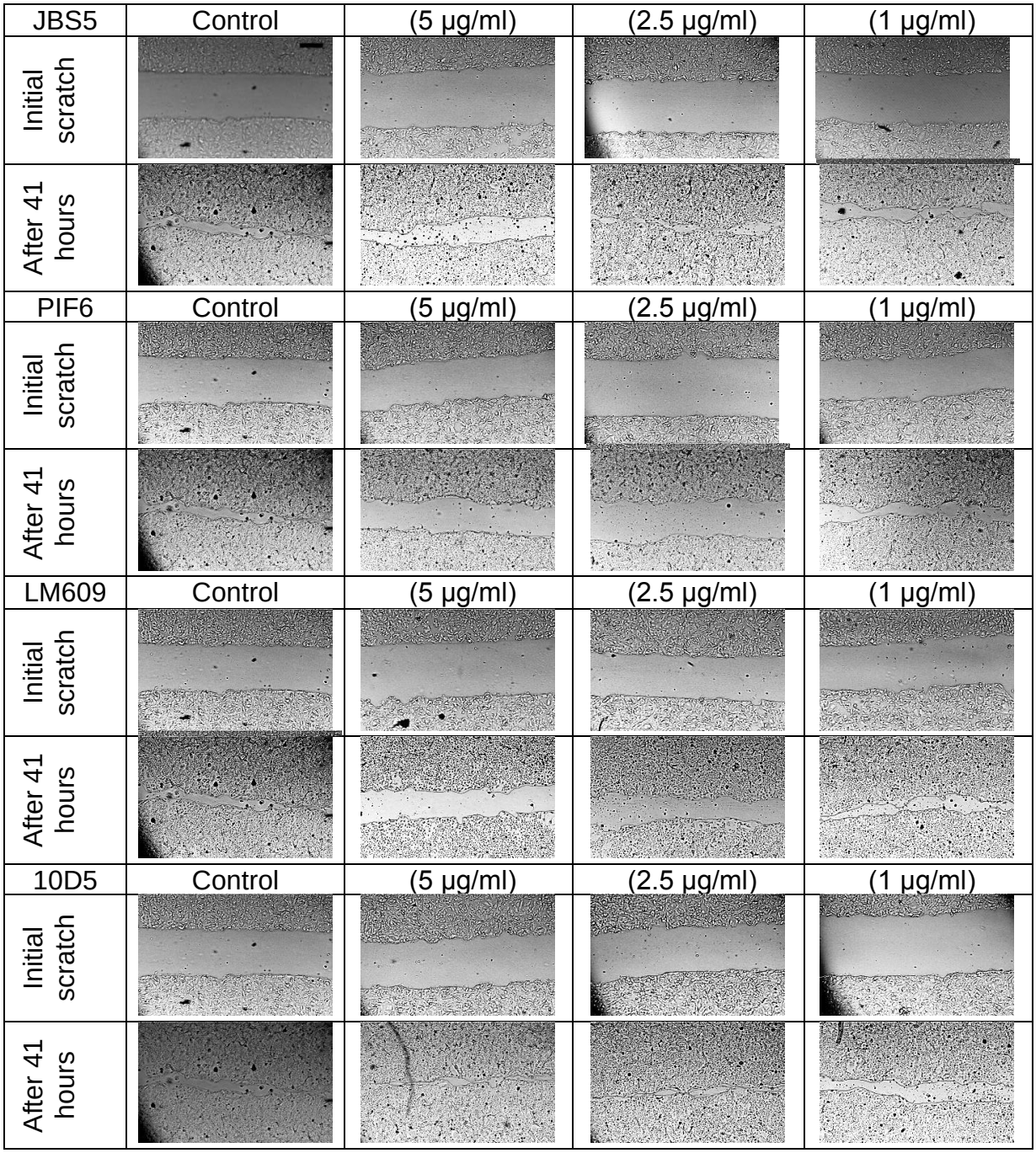


Figure 43 The effects of integrin receptor blocking with neutralizing antibodies on DLD-1 cell migration.

DLD-1 cells were treated with different concentration of LM609 (anti-αvβ3), PIF6 (anti-αvβ5), JBS5 (anti-α5β1) and 10D5 (anti-αvβ6). PIF6 and LM609 antibodies were the most effective in inhibiting cell migration. Bar length = 200 µm.

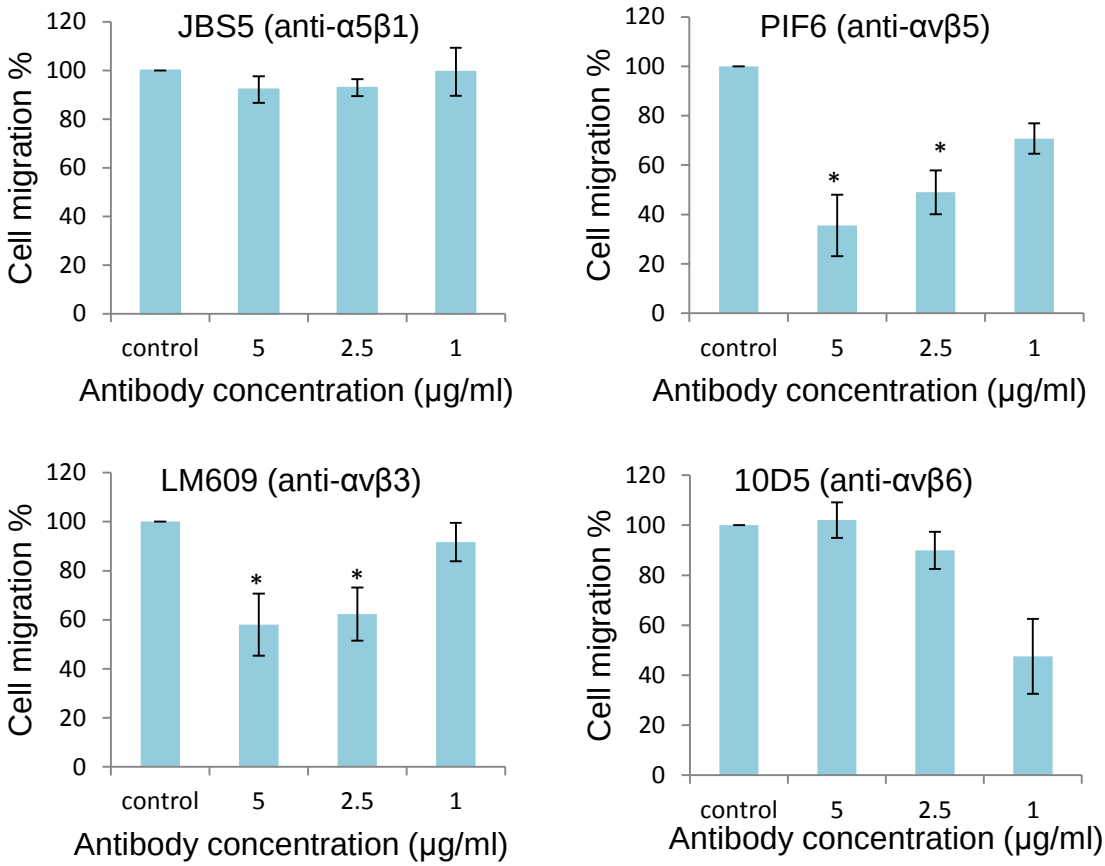


Figure 44 The effect of integrin receptor blocking with neutralizing antibodies on DLD-1 cell migration.

Results shown average ± SD of three independent experiments. The asterisk * indicates $P < 0.05$ and ** indicates $P < 0.01$.

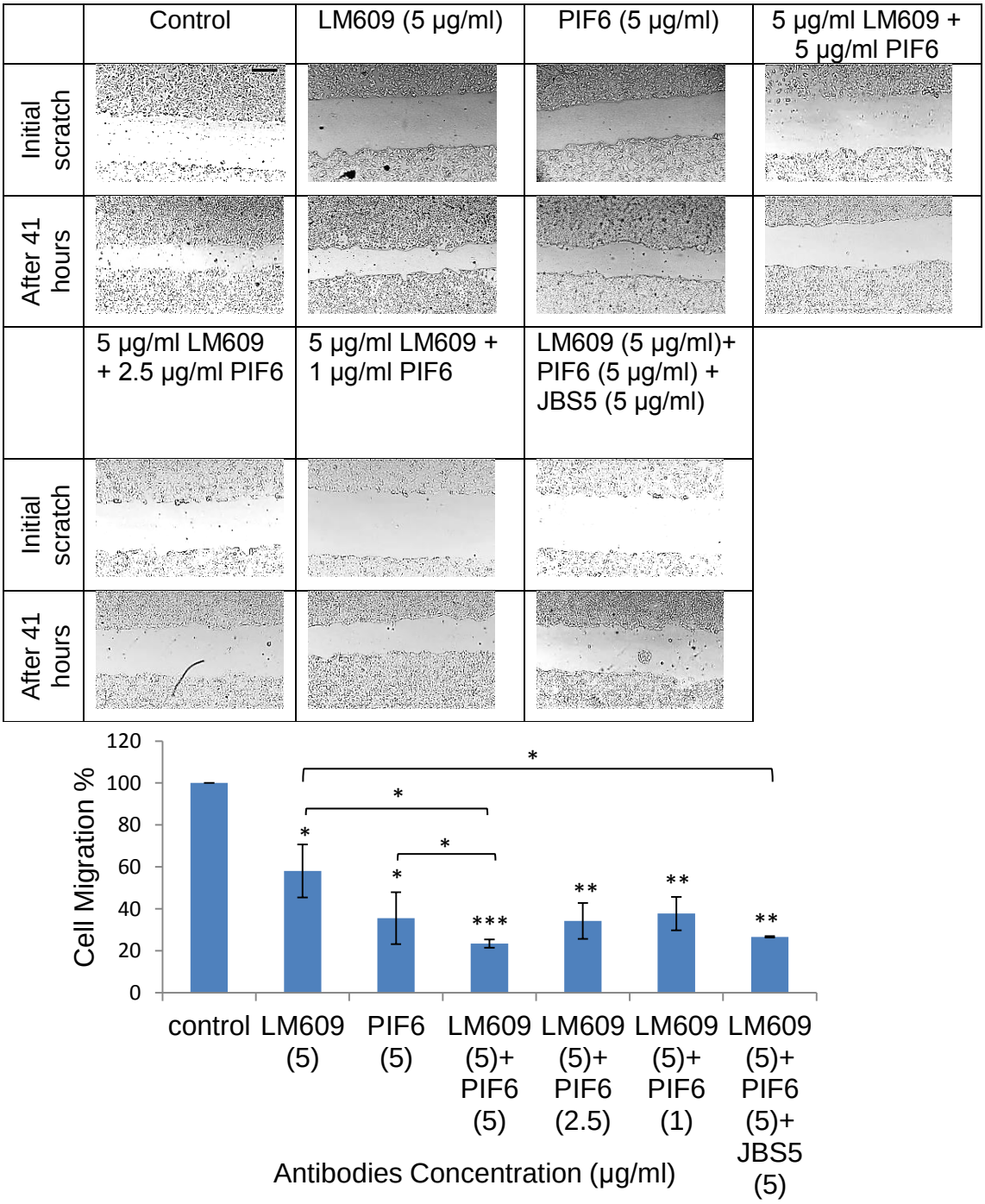


Figure 45 The effect of combination anti-integrin treatment on DLD-1 cell migration.

DLD-1 cells were treated with two or more antibodies. Scale bar = 200 µm. Results shown are representative/averages of three independent experiments. The asterisk * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

4.4.1.1.5 **Effect of novel small molecule antagonists on DLD-1 cell migration.**

The non-cytotoxic concentrations of integrin antagonists were evaluated by MTT assay. The MTT assay is a colourimetric method where viable cells with dehydrogenase activity metabolise MTT (a yellow water-soluble tetrazolium salt; 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide) into a purple coloured formazan product. Mitochondrial succinate dehydrogenase is the principle reductase involved. Dead cells are unable to convert MTT into formazan thus colour formation is proportional to the number of viable cells. DMSO is used to dissolve the formazan product formed and the absorbance of the resulting solution is measured by vis-spectrophotometry. The intensity of the product colour is measured at 550 nm, and the optical density is directly proportional to the number of living cells in the culture.

The MTT assay was used to evaluate the cytotoxicity of 7 novel small molecule integrin antagonists which were synthesised at the ICT (ICT9055, ICT9072, ICT9073, ICT9085, ICT9087, ICT9088 and ICT9094) against the DLD-1 cell line (expresses high level of αvβ5). The cytotoxicity of ICT9019, ICT9023, ICT9024 and ICT9026 compounds had previously been established in the DLD-1 and DU145 cell lines (Unpublished results, M Sutherland). Non-cytotoxic concentrations of each compound (IC_{20}) defined as a dose that kills less than 20% of cells was obtained from MTT dose-response curves (Figure 46).

Non-cytotoxic concentrations IC_{20} (summarized in Table 23) were used in scratch assay to evaluate the effect of these compounds on DLD-1 cell migration. ICT9055 and ICT9072 inhibited the cell migration by 70% and 60%

respectively. Other compounds had a negligible effect on the migration of cells (Figure 47).

ICT9055 and ICT9072 are known to have high activity against αvβ3 integrin (Alshammari, 2013) but in this study, these two compounds significantly inhibited the migration of DLD-1 cells. As shown in Figure 47, both αvβ3 and αvβ5 contributed significantly to DLD-1 migration, and contribution of αvβ5 may be slightly higher than αvβ3. To investigate the specificity of ICT9055 and ICT9072, the scratch assay cells were incubated with LM609 antibody to block αvβ3 whilst allowing binding to αvβ5. Cells were then incubated with ICT9055 and ICT9072. I found that blocking of αvβ3 integrin increased the effect of these compounds. ICT9055 with or without LM609 inhibited the cell migration by 80% and 70% respectively. Also ICT9072 with or without LM609 inhibited the cell migration by 85% and 45% respectively (Figure 48). Incubating the cells with LM609 and compound together was significantly more effective than sequential treatments with 2 separate agents. Taken together with previous results, these data could indicate that ICT9072 and ICT9055 are inhibitors of both αvβ5 and αvβ3 integrins or that LM609/compound alone incompletely inhibits αvβ3 at the concentration tested whereas the combination improves receptor occupancy. LM609 and the compounds could displace one another from integrins.

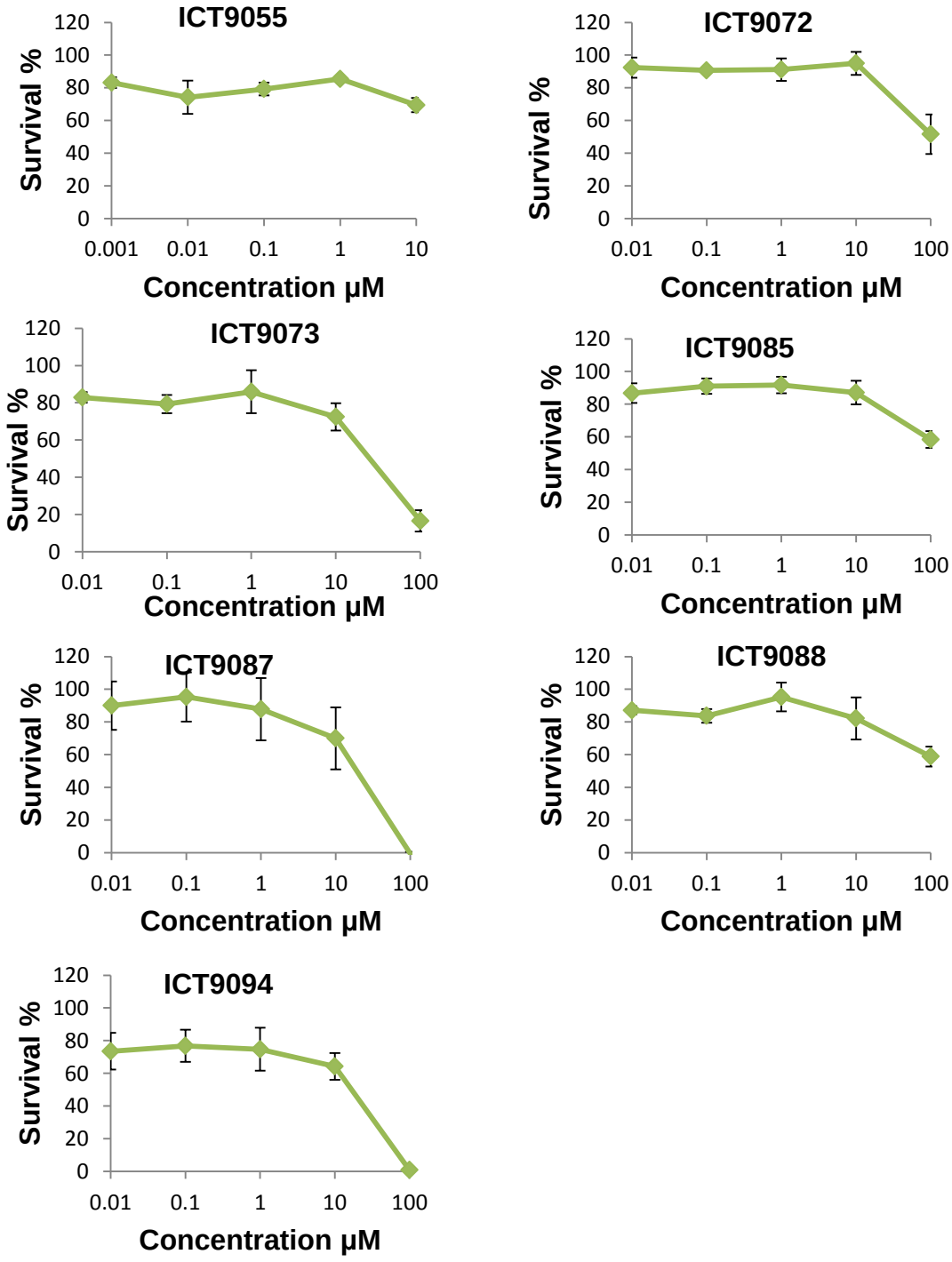


Figure 46 Dose response curves of the cytotoxicity of small molecule integrin antagonists in DLD-1 cells.

The curve profiles were obtained by three *in vitro* experiments. Mean ± SD of triplicate determination is given.

Compound	Non-cytotoxic concentrations used on DLD-1 IC_{20} (µM)	IC_{50} (µM)
ICT9019	5	> 5
ICT9023	100	> 100
ICT9024	5	100
ICT9026	100	> 100
ICT9055	5	> 10
ICT9072	10	100
ICT9073	1	25
ICT9085	10	> 100
ICT9087	1	18
ICT9088	10	> 100
ICT9094	1	18

Table 23 Non-cytotoxic concentrations of novel small molecule integrin antagonists which were synthesised at the ICT

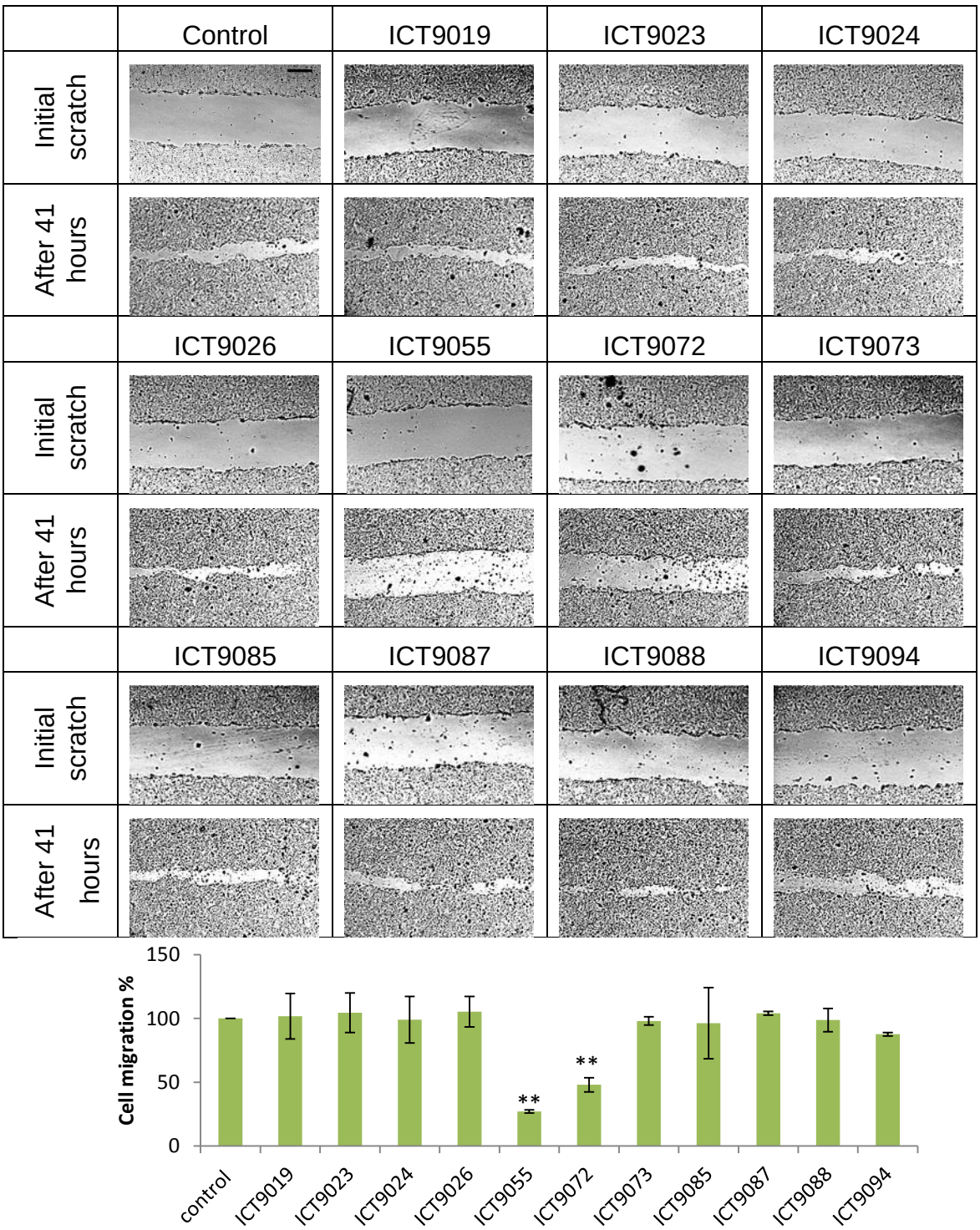


Figure 47 The effect of novel small molecule integrin antagonists on DLD-1 cell migration.

DLD-1 cells were incubated with small molecule integrin antagonists for 41 hours. Bar length = 200 μm. Results shown are representative/average of three independent experiments. The asterisk * indicates $P < 0.05$ and ** indicates $P < 0.01$.

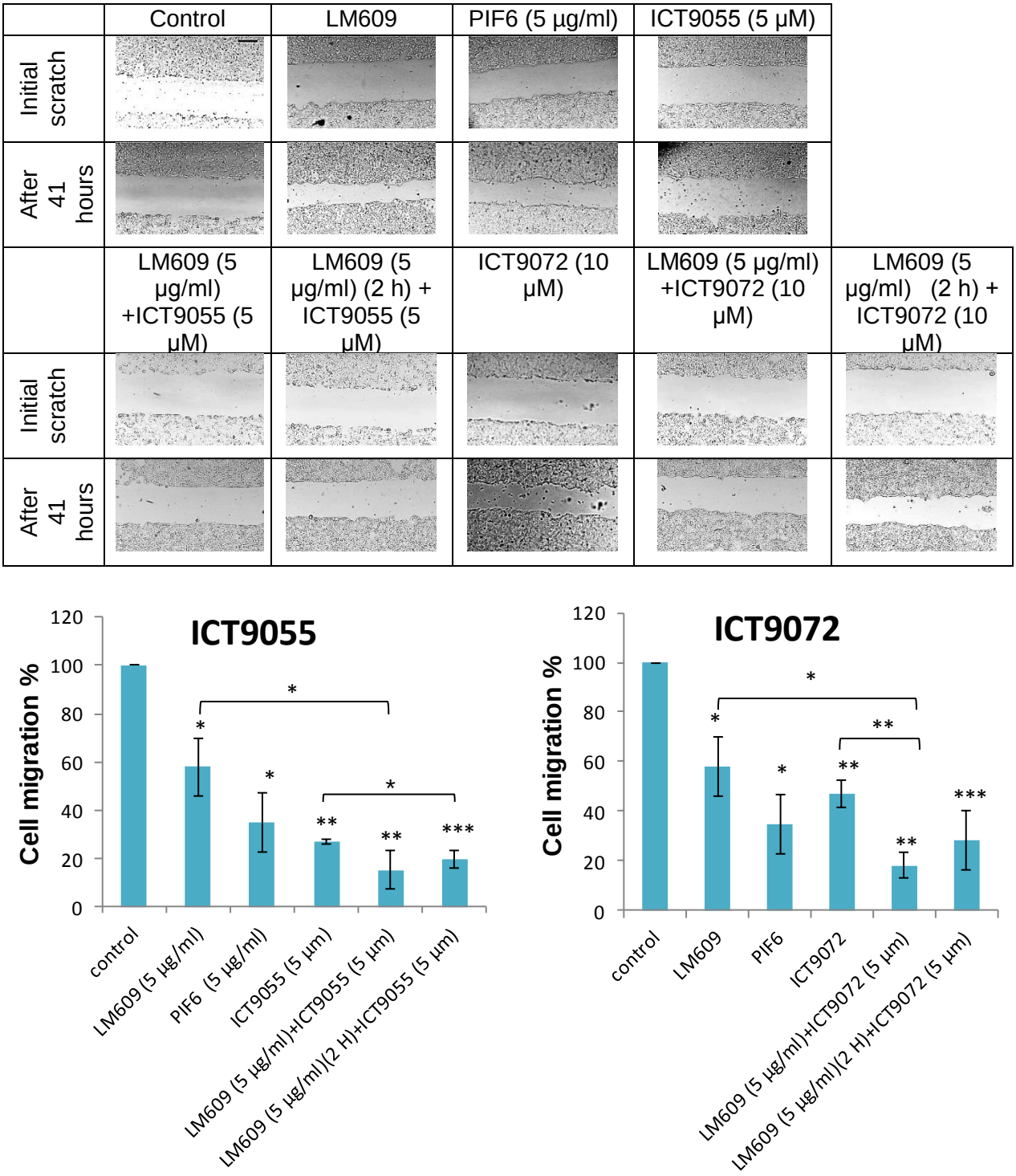


Figure 48 Evaluation of the specificity of ICT9055 and ICT9072.

DLD-1 cells were incubated with LM609 or PIF6 or with a mixture of LM609 and ICT9055 or ICT9072, or preincubated with LM609 for 2 hours before compounds were added. Results shown are representative of three independent experiments. Scale bar = 200 µm. The asterisk * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$.

4.4.1.1.6 **Determination of which integrins control migration in DU145 cells**

The DU145 cell line (which expresses αvβ3 and αvβ5 integrins) was used as a model to evaluate dual antagonists on cell migration. The same antibodies used in DLD-1 except 10D5 (anti-αvβ6) were used to characterise the DU145 cell line in the scratch assay to determine which integrins have significant role in DU145 cell migration.

The results show that both αvβ3 and αvβ5 integrins had a significant role in DU145 cell migration; PIF6 and LM609 antibodies both inhibited the cell migration by 45 % and this is less effective than in DLD-1, and the dose response curves are flat. JBS5 antibody had a reverse dose-response compared to PIF6 and LM609 antibodies effect on cell migration where the lowest concentration inhibited the cell migration by 30% but the highest concentration had no effect (Figure 49 and Figure 50).

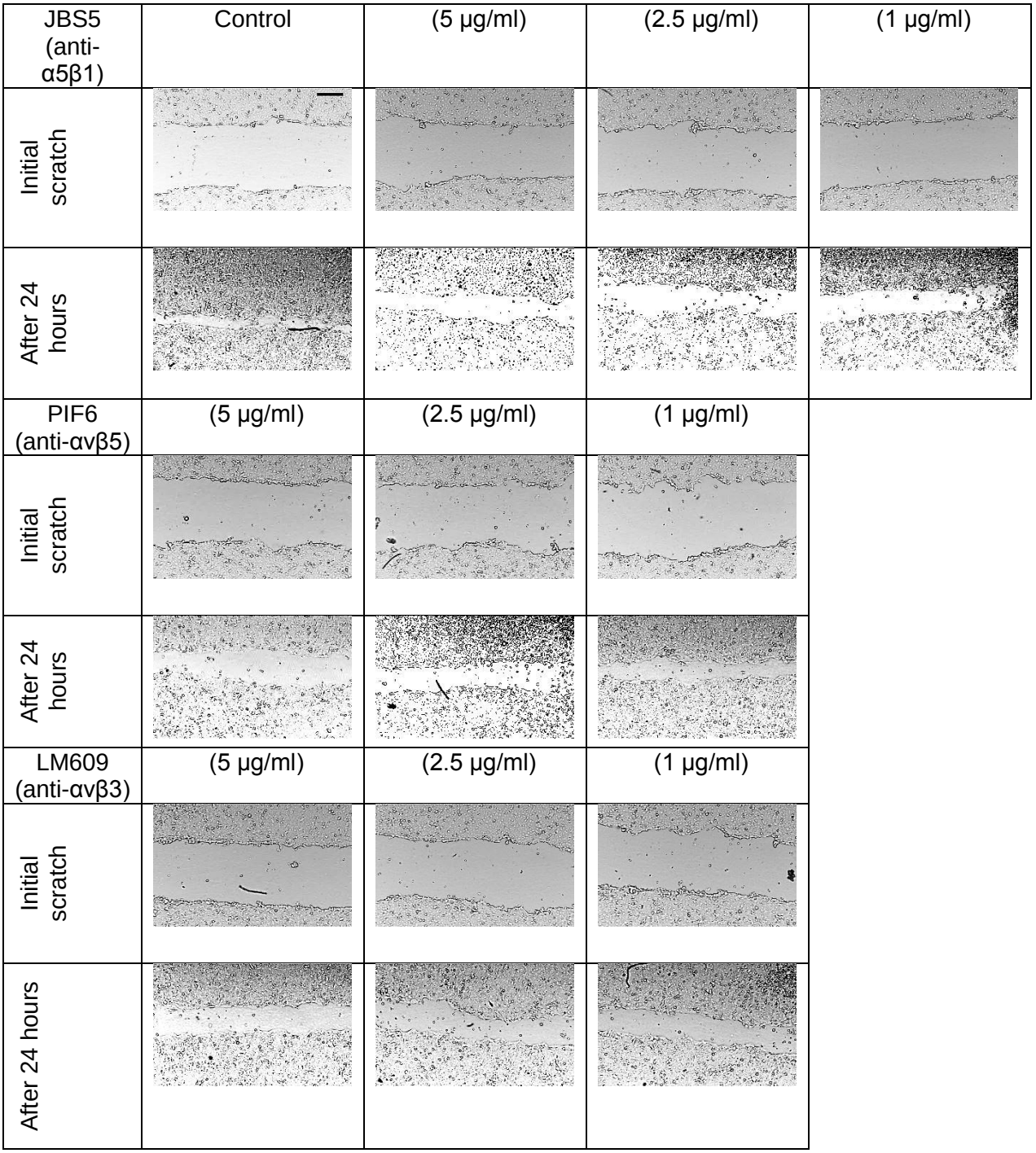


Figure 49 The effect of integrin receptor blocking with inhibitory antibodies on DU145 cell migration.

DU145 cells were treated with different concentration of LM609 (anti-αvβ3), PIF6 (anti-αvβ5) and JBS5 (anti-α5β1). PIF6 and LM609 antibodies were the most effective to inhibit cell migration. Bar length = 200 µm.

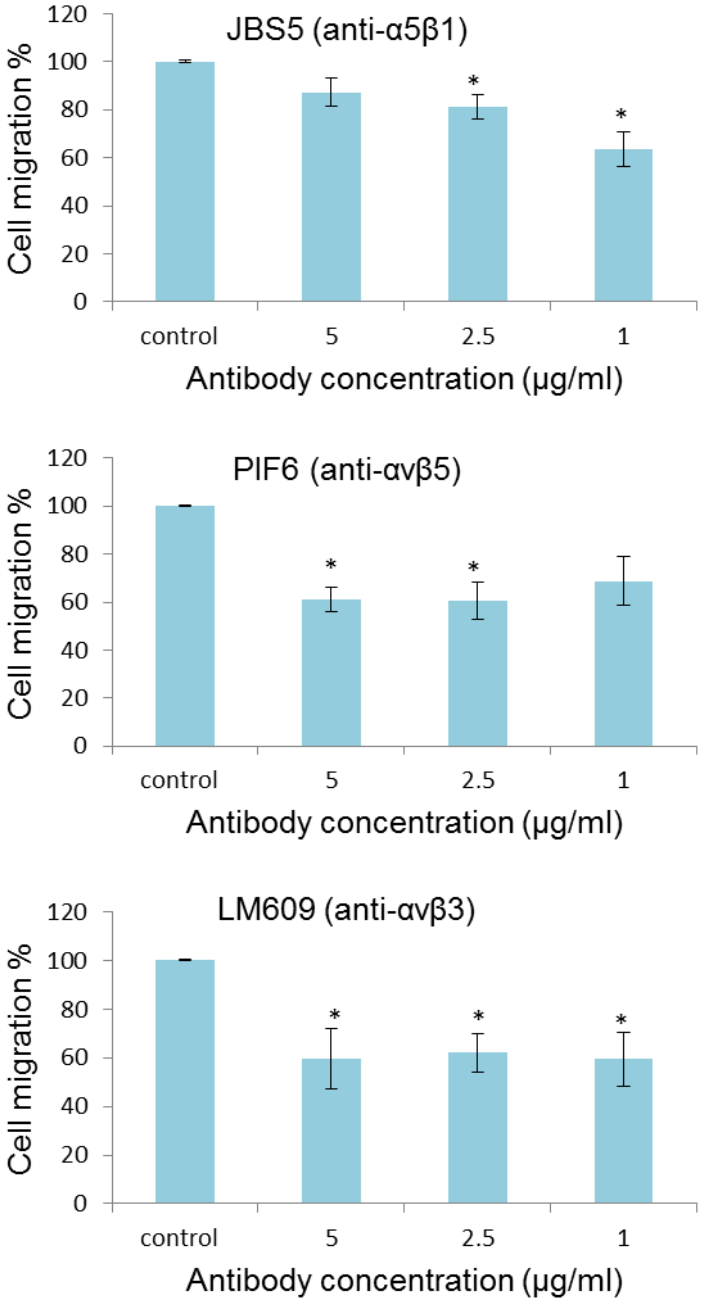


Figure 50 The effect of integrin receptor blocking with neutralizing antibodies on DU145 cell migration.

DU145 were scratched and treated with different concentrations of each antibody (LM609 (anti-αvβ3), PIF6 (anti-αvβ5) and JBS5 (anti-α5β1)). Results shown are average ± SD of three independent experiments. * indicates $P < 0.05$.

4.4.1.1.7 **The effect of novel small molecule antagonists on DU145 cell migration.**

The non-cytotoxic concentration IC_{20} and IC_{50} of each compound was obtained from the respective MTT curve (Figure 51) and summarized in Table 24. The non-cytotoxic concentrations IC_{20} were used in the scratch assay to evaluate the effect of these compounds on cell migration.

ICT9024, ICT9026 and ICT9023 (50 µM) and ICT9087 (1 µM), significantly decreased cell migration by 60%, 60%, 50% and 20% respectively. ICT9085 and ICT9094 (10 µM) and ICT9073 (0.1 µM) inhibited cell migration by 30%. At the lowest concentration (0.01 µM), ICT9055 inhibited cell migration by 25%. Other compounds had a negligible effect on the migration of cells (Figure 52). This suggests that ICT9024, ICT9026, ICT9023 and ICT9087 can block either or both αvβ3 and αvβ5 integrins thus inhibiting DU145 cell migration.

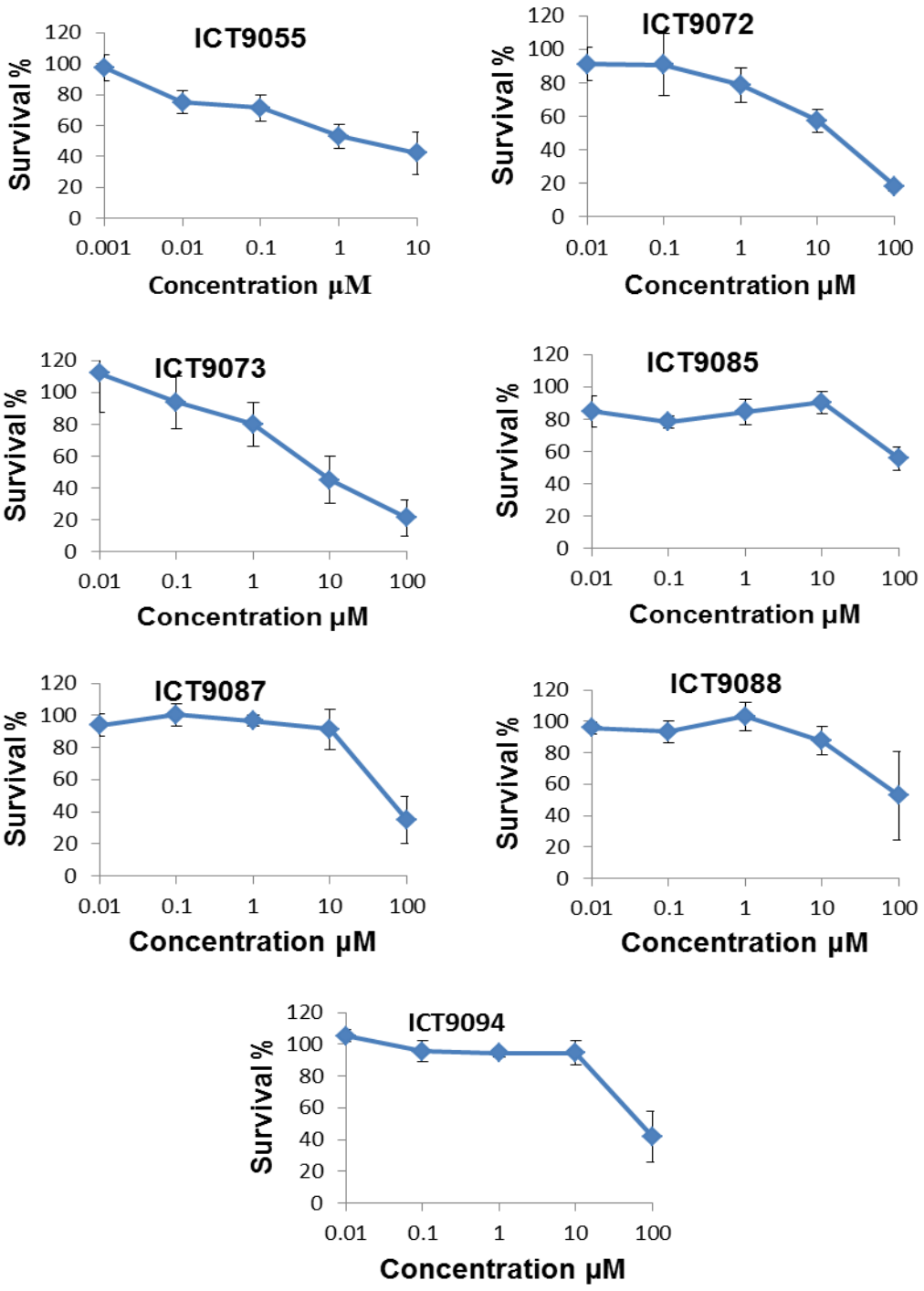
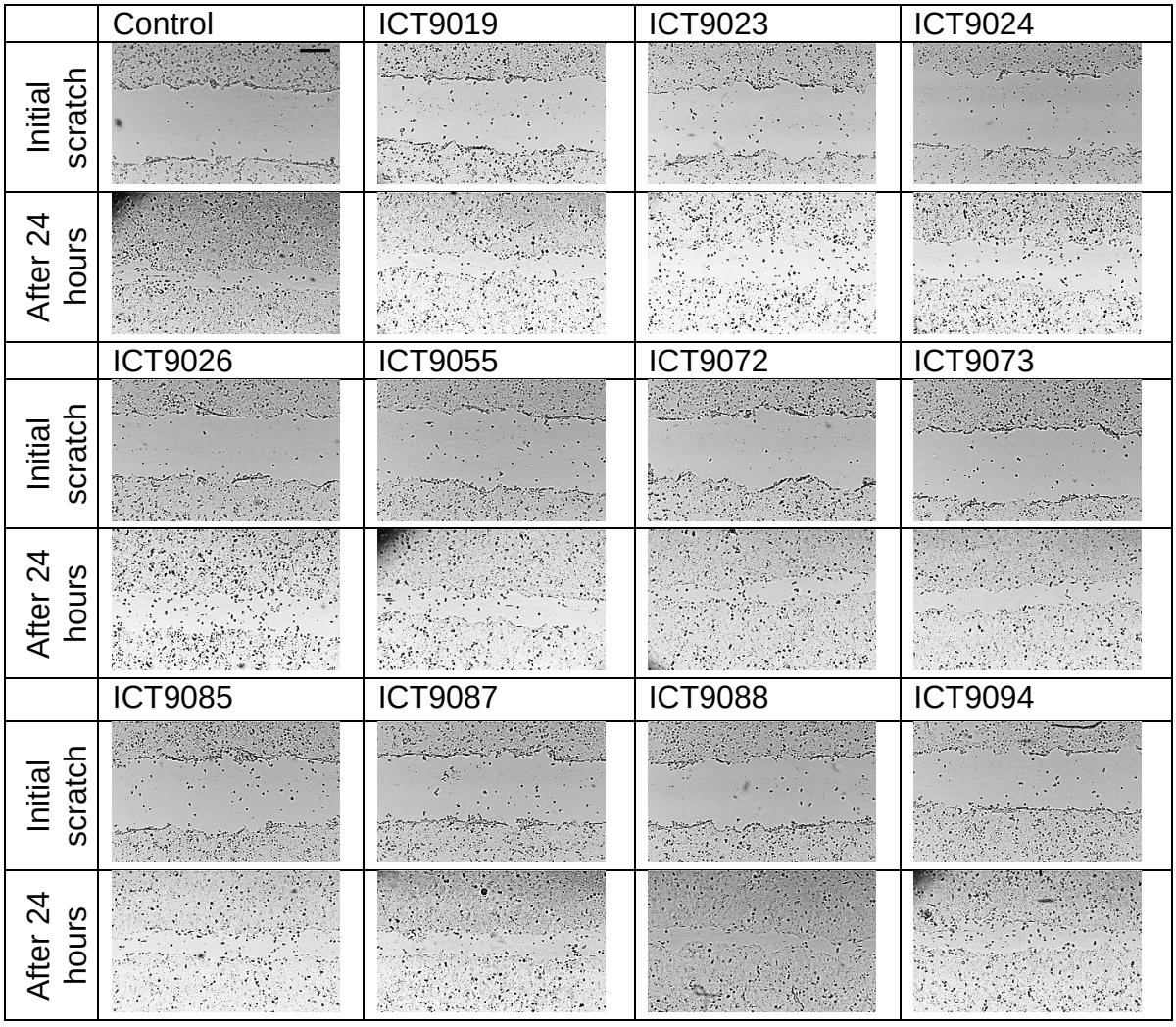


Figure 51 Dose response curves of the cytotoxicity of small molecule integrin antagonists in DU145 cells.

Mean ± SE of triplicate determination is given.

Compound	Non-cytotoxic concentrations used on DU145 IC_{20} (µM)	IC_{50} (µM)
ICT 9019	5	100
ICT9023	50	> 100
ICT9024	50	> 100
ICT9026	50	> 100
ICT9055	0.01	1
ICT9072	0.1	17
ICT9073	0.1	8
ICT9085	10	> 100
ICT9087	1	50
ICT9088	1	100
ICT9094	10	70

Table 24 Non-cytotoxic concentrations of novel small molecule integrin antagonists which were synthesised at the ICT



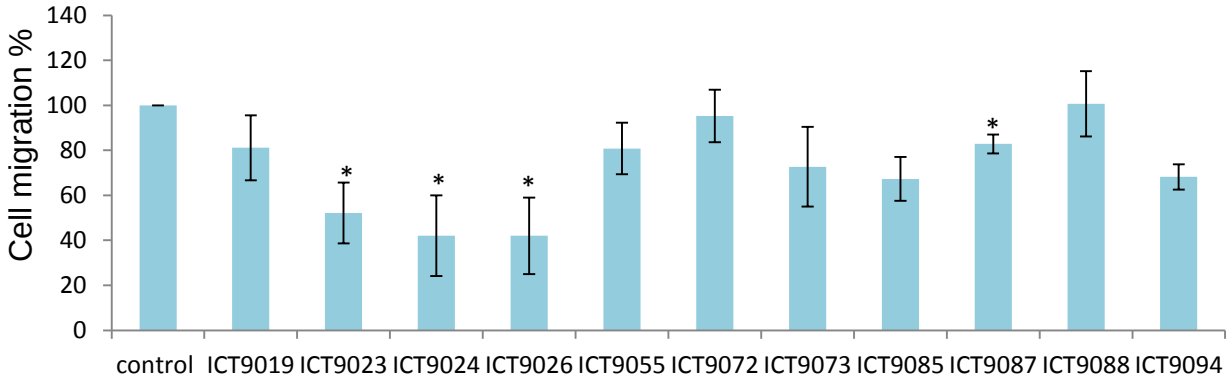


Figure 52 The effect of novel small molecule integrin antagonists on DU145 cell migration.

DU145 cells were wounded and then incubated with different concentrations of integrin antagonists for 24 hours. Bar length = 200 μm. Results shown are representative/average of three independent experiments. * indicates $P < 0.05$.

4.4.1.1.8 **Determination of which integrins control migration in M14 cells**

Previous results in our group showed that blocking αvβ3 integrin by LM609 (2.5 μg/ml) resulted in 55% inhibition of M14 cell migration. In this study the participation of αvβ5 and α5β1 integrins in M14 cell migration was evaluated also by scratch assay. M14 cell migration was 50% inhibited by integrin receptor blocking with PIF6 (2.5 μg/ml). JBS5 antibody (2.5 μg/ml) increased migration by 40 % (Figure 53). The integrin expression profile showed that JBS5 antibody increased the expression of αvβ3 and induced ectopic expression of αIIbβ3 (Figure 40).

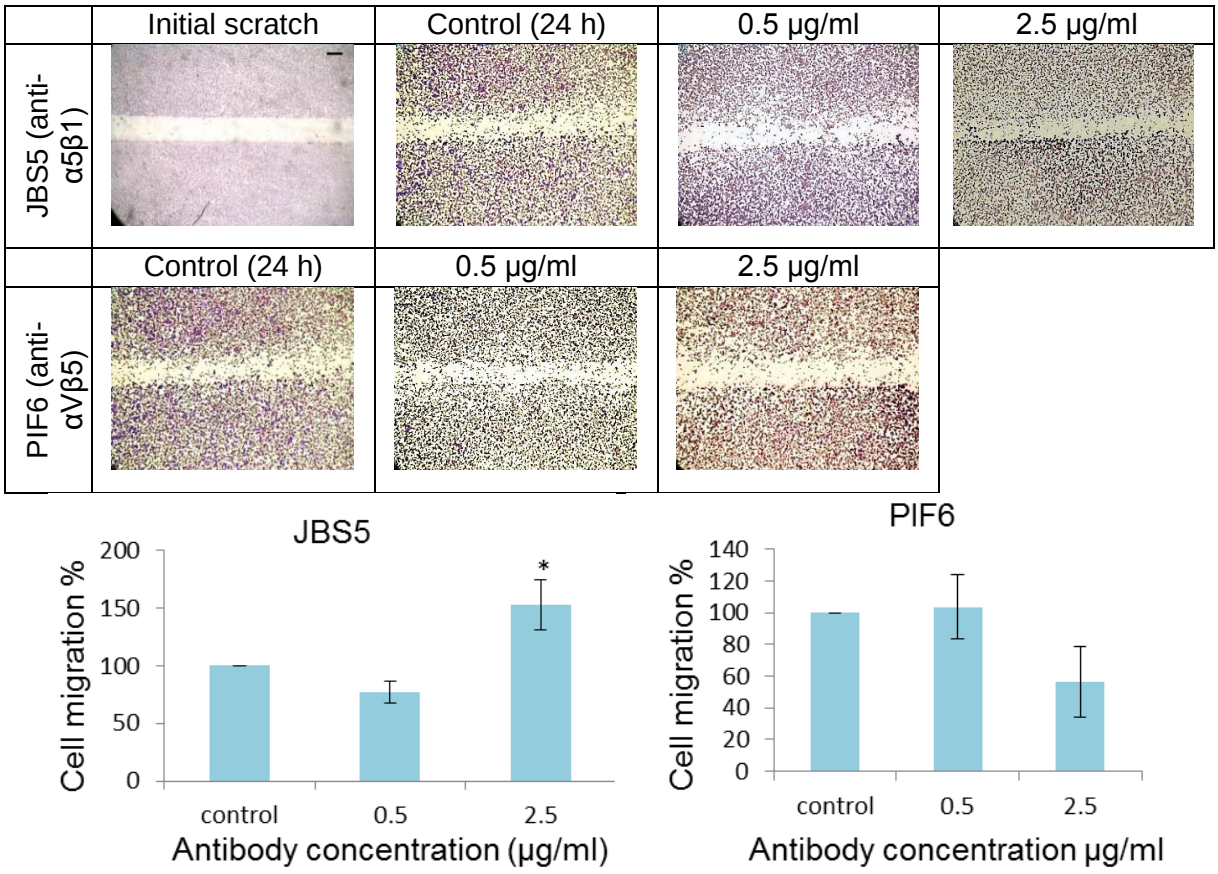


Figure 53 The effect of integrin receptor blocking on M14 cell migration.

Results are shown as representative/average of three independent experiments. * indicates $P < 0.05$. Bar length = 200 μm.

4.5 DISCUSSION

The objective of this study was to develop models that can be used to evaluate the potential of new integrin antagonists on inhibiting cancer cell migration. In order to test this, the blockade of one or multiple integrins by neutralizing antibodies or small molecule antagonists was performed in a 2D wound-healing assay (scratch assay).

In a wound-healing assay, the cells used should have certain characteristics, such as the ability to form a monolayer, the ability to be motile, and prolonged doubling times. These characteristics were studied among the cell line models selected in Chapter 3. I found that MCF-7 (expressing a high level of the αvβ5 integrin and a lack of the α5β1 integrin) and MCF-7ADR were unable to form monolayers because they grow as clumps. Furthermore, the HT-29 cell line was found to be unsuitable for use in a wound-healing assay because it does not have the ability to be motile and migrate on a plastic surface. In support of this finding, Herrera found that HT-29 cells do not spread on plastic surfaces unless a hepatocyte growth factor is added (Herrera, 1998).

Other cell line models, such as DLD-1, OSC-19, PC-3, M14, and DU145, were found to form a monolayer and migrate. The DLD-1, OSC-19, and DU145 cell lines showed sheet-like epithelial migration behaviour (collective migration), whereas, the M14 and PC-3 cell lines migrated as single cells. These cell line models were screened in terms of wound healing time in order to select the best model. The OSC-19 cells showed rapid wound healing (with a scratch nearly 90% healed after 18 hours). The PC-3, M14, and DU145 cells exhibited moderate wound healing after 24 hours, whereas DLD-1 showed slow wound healing

(with a scratch nearly 90% healed after 41 hours). In prolonged wound healing, cells with short doubling time make distinguishing between cell proliferation and migration is difficult. Therefore, the expression of proliferation marker Ki67 was evaluated by immunofluorescence. In the DU145 and DLD-1 cells, the cells at the scratch edges expressed low levels of Ki67, which meant that cells moving into the wound area had migrated.

Moreover, this study confirms that different concentrations of serum have no effect on the rate of cell migration, although they could have an effect on cell proliferation. This observation is in agreement with the results of Kim et al. (2012), who observed the same cell migration rates in mediums with 5% and 10 % FBS solutions (Kim et al., 2012).

The OSC-19 cell line expresses high levels of αv, β5 and α5 integrin subunits and this is in agreement with the expression profile of integrins in clinical samples where the tumour cells express high and moderate level of β5 and α5β1 integrins respectively. The exploration of integrin antagonist properties in this cellular model may reflect what could happen when blocking several integrins in cell migration. The contribution of α5β1, αvβ3, and αvβ5 to cell migration was studied in specific antibody-blocking experiments. As expected, αvβ5 proved to be essential for the ability of the cells to migrate. Bianchi *et al*. demonstrated that β5 plays a crucial role in the TGFβ-induced epithelia mesenchymal transition. Further they found that depletion of β5 integrin significantly inhibited breast cancer cells invasion (Bianchi et al., 2010a). αvβ5 integrin also enhances cell migration and liver metastasis of colon carcinoma mediated by hepatocyte-derived heregulin (Yoshioka et al., 2010).

Blocking either αvβ3 or αvβ5 inhibited cell migration by 25% and 30%. Surprisingly, the combination of αvβ3 and αvβ5 anti-integrin antibodies did not increase the inhibitory effect, which might mean that blocking both αvβ3 and αvβ5 releases signalling effectors for use by another integrin (Parikka et al., 2006). Crosstalk between integrins plays a role in many biological processes, such as tumour invasion, morphogenesis, and metastasis, and includes the combination or activation of integrins that stimulate or inhibit the function of other integrins in the same cell. Many studies have proven the crosstalk between integrins such as β1, β2, and β3 (Pijuan-Thompson and Gladson, 1997) (Simon et al., 1997). For example, the activity of the α2β1 integrin receptor is stimulated by αvβ5/6 integrins in colon cancer (Defilles et al., 2009).

My data showed that blocking α5β1 integrin enhances OSC-19 cell migration. This study showed that blocking the α5β1 integrin induces the alteration and switching of the expression of other integrins. Protein expression profiling revealed marked changes in αIIbβ3 integrin levels that may be implicated in OSC-19 cell migration. This result is similar to the work of Parvani *et al*. who showed that inactivation of β1 integrin by inhibitory antibodies leads to increased expression of β3 by activating p38 MAPK pathway, which in turn enhanced the metastasis of breast cancer (Parvani et al., 2013). Moreover, our data accords quite well with a study by Jeanes *et al*., who deleted the β1-integrin gene *in situ* from primary cultures of mammary epithelial cells to study the cellular role of specific integrins in cell proliferation and migration. The study found that either the knockdown or artificial inhibition of β1-integrins in breast cancer enables β3 integrin subunits to promote migration or even metastases

(Jeanes et al., 2012). In further agreement with these results, a study by Vellon *et al*. found the blockage of α5β1 integrins induced the cell migration of hepatic progenitor cells by stimulating cell-signalling pathways involved in cell motility (Vellon et al., 2010).

I found inhibition by the αvβ3 and αvβ5 integrin antibodies to be greater in the presence of the α5β1 antibody and αIIbβ3 small molecule antagonists, indicating that multiple integrins participate in OSC-19 cell migration, and complete blockade of both β3 integrins is required to reverse the pro-migratory effects of α5β1 inhibition. Our findings suggest that the selective deactivation of the α5β1 integrin may not be a safe therapy for head and neck cancer, whereas multi-integrin targeting may play a significant role in inhibiting head and neck cancer metastasis. This result provides a new interpretation of the results of the Phase II clinical trial of cilengitide in combination with cisplatin, 5-fluorouracil, and cetuximab did not improve progression-free survival in patients with recurrent and/or metastatic of head and neck squamous cell carcinomas (Vermorken et al., 2014); cilengitide is a dual αvβ3 and αvβ5 inhibitor which also has some effects on α5β1 integrin. This anti-α5β1 activity may induce αIIbβ3 expression, providing a resistance mechanism since cilengitide does not inhibit αIIbβ3. Still this data needs confirmation by using other head and neck cancer cell lines and α5β1 integrin antibodies to exclude an idiosyncratic effect.

In this study, the DLD-1 cell line was utilised because it expressed moderate levels of α5 and β5 and a low level of β3. The contribution of the α5β1, αvβ3, αvβ5, and αvβ6 integrins in DLD-1 cell migration was determined in specific

antibody blocking experiments. The data showed that DLD-1 cell migration was mediated primarily by the αvβ5 integrin and partially by αvβ3. This result is in agreement with Enns *et al*. who demonstrated that colon cancer cells attached to vitronectin mainly by the αvβ5 integrin and blocking the αvβ5 integrin by antibodies or small molecules inhibits cell adhesion to vitronectin, which enables the metastasis of cells (Enns et al., 2005a).

Moreover, the data showed that α5β1 integrin was not essential in DLD-1 cell migration, and its blockage did not affect cell migration. Surprisingly, the lowest concentration of αvβ6 inhibited cell migration by 50%, whereas the highest concentration had no effect. It has been shown that αvβ6 integrin plays an important role in colon cancer progression where its expression lead to increased cell proliferation and enhanced cell migration and invasion (Cantor et al., 2013) mediates metastasis to the liver (Yang et al., 2008) and stimulation of MMP-9 (Gu et al., 2002). The unexpected results in our case could be due to the ability of αvβ6 integrin to release cell signalling effectors to interact with other integrins and enhance their function. A study by Defilles *et al*. provided evidence for the negative regulation of the α2β1 integrin by the αvβ5/β6 integrins in two different adenocarcinoma cell lines. The blocking of αvβ5/β6 integrins was found to enhance cell migration toward type I collagen, and α2β1 engagement was found to enhance outside-in signalling by increasing the phosphorylation of FAK and ERK1/2 (Defilles et al., 2009). In addition, Cantor *et al*. showed that transfection of SW480 colon cancer cells with β6 repressed the expression of other integrins whose expression is known to have a role in cancer progression such as α2, α6, β1, β4 and β5. Therefore, inhibition of β6

could lead to expression and enhanced cell migration dependent on other integrins (Cantor et al., 2013).

After characterizing the assay and identifying which integrins were primarily responsible for DLD-1 cell migration, the effect of novel small molecule antagonists on DLD-1 cell migration was evaluated. Before using the antagonists on DLD-1 cells, the cytotoxicity of certain compounds was tested using the MTT assay. The cytotoxic effect was evaluated to confirm that the results obtained were due to blockade of integrin function (inhibition of cell migration in the scratch assay), not due to general toxicity.

ICT9023 and ICT9026 had no toxic effects on DLD-1 cells whereas, other compounds had variable toxic effects on DLD-1 cells and this may be due to the inhibition of αvβ5 or αvβ6 integrins which are known to be important in colon cancer survival. O'Brien *et al*. showed that αvβ5 suppress the apoptosis of colon carcinoma cells induced by serum deprivation and it could be that these compounds inhibit αvβ5 receptor and mediate apoptosis (O'Brien et al., 1996). Also it has been shown that αvβ6 integrin is involved in tumour growth and down regulation of this receptor inhibits tumour growth *in vivo* (Ahmed et al., 2002). Zhao *et al*. showed that blocking αvβ6 integrin caused apoptosis of colon cancer *in vitro* (Zhao-Yang et al., 2008).

Among these compounds, the highest effect of inhibiting DLD-1 cell migration came from ICT9055 and ICT9072. According to previous unpublished ELISA and adhesion assay results (Fatemah Alshammar, Mark Sutherland, Andrew Gordon and Manar Zraikat), ICT9055 has shown high activity against the αvβ3 integrin. However, DLD1 expressed several αv integrins. To determine whether

the effect on DLD1 was due to inhibition of αvβ3, αvβ5 or both, cells were preincubated with the LM609 antibody (5 μg/ml) to block the αvβ3 integrin before treatment with ICT9055 or ICT9072. I found blocking the αvβ3 integrin to increase the effect of ICT9055 and ICT9072. These data may indicate that these two compounds have affinity for the αvβ5 integrin as well as αvβ3, and further suggests that the two heterodimers cooperated in the regulation of DLD-1 cell migration. Alternatively this result could indicate that blocking of integrin by either LM609 or compound incompletely inhibits αvβ3 whereas the combination improves receptor occupancy. LM609 and compound could displace one another from integrins, which further complicates interpretation. It is important to study the selectivity of the compounds on cells that express single integrins but in this study it was difficult to find cell lines that express one integrin. Therefore, it is worthy to do integrin knockdown or transfection studies in the future.

In addition, due to its expression of high levels of the β5 and α5 integrin subunits, the DU145 prostate cancer cell line was used to evaluate the effect of novel small molecule antagonists in the scratch assay. Characterisation with blocking antibodies showed that the αvβ5 and αvβ3 integrin antibodies have the same effect on DU145 cell migration, significantly inhibiting cell migration by 40%. Studies have shown that prostate cancer cells adhere to vitronectin by the αvβ3 integrin (Zheng et al., 1999) and migrate on vitronectin by αvβ5 (Bisanz et al., 2005). Also it has been shown that blocking integrins αvβ5, α5β1, and αvβ3 by a disintegrin contortrostatin inhibits the migration of PC-3 prostate cancer cells (Lin et al., 2010). Furthermore, Witkowski *et al*. found DU145 to depend on

the α5β1 integrin to adhere to fibronectin (Witkowski et al., 1993), and Stachurska *et al*. have reported the α5β1 integrin to enhance the adhesion and migration of prostate cancer cells (PC-3) to fibronectin (Stachurska et al., 2012). In agreement with this, I found the α5β1 integrin to play a crucial role in DU145 cell migration in that the α5β1 antibody showed a reverse dose response. The lowest concentration of α5β1 antibody significantly reduced cell migration, whereas the highest concentration did not affect cell migration. This could be due to the fact that the complete inhibition of the α5β1 integrin activates intracellular signalling to regulate cell functions by another integrins; if α5β1 is not able to signal, intracellular signalling effectors are redirected to other integrins, enhancing their signalling. Indeed, crosstalk between α5β1 and other integrins has been well documented. Kim *et al*. found the α5β1 integrin to regulate the αvβ3 integrin and mediate endothelial cell migration and angiogenesis (Kim et al., 2000b), and Lishko *et al*. demonstrated increasing levels of integrin αMβ2 to retard α5β1-mediated migration (Lishko et al., 2003).

Among the tested compounds, ICT9023, ICT9024, ICT9026 and ICT9087 had a significant effect on inhibiting DU145 cell migration. These compounds appear to have effect on multiple integrins including αvβ3, αvβ5 and especially α5β1 integrin which has been shown to participate in DU145 cell migration but not in DLD-1 cell migration. ICT9085, ICT9094, ICT9019, ICT9087, ICT9073, and ICT9055 had a moderate effect on cell migration (about 20%). ICT9072 showed no effect on DU145 cell migration and this is in contrast to its effect on DLD-1 cell migration. This could be due to an effect on αvβ6 integrin which is absent in DU145 (in house PCR data, Andrew Gordon). The

comparison of the effect of ICT compounds on DLD-1 and DU145 cells migration is summarized in Table 25.

	DLD-1	DU145
Protein expression profile	αvβ3, αvβ5 and α5β1	αvβ3, αvβ5 and α5β1
RNA expression profile	αvβ6	Express neither αvβ6 nor αvβ8
Integrin which primarily responsible for tumour cell migration	αvβ5, αvβ3 and αvβ6	αvβ5, αvβ3 and α5β1
Compound number	Inhibition percentage	Inhibition percentage
ICT9019	No effect	20%
ICT9023	No effect	50%
ICT9024	No effect	55%
ICT9026	No effect	55%
ICT9055	80%	20%
ICT9072	60%	No effect
ICT9073	No effect	20%
ICT9085	No effect	22%
ICT9087	No effect	20%
ICT9088	No effect	No effect
ICT9094	20%	20%

Table 25 Comparison of the effect of ICT compounds on migration of DLD-1 and DU145 cells

In previous work (Alshammari, 2013), the melanoma cell line M14 was used as a αvβ3 integrin model to evaluate the effect of novel small molecule antagonists on cell migration. The results showed the αvβ3 integrin to play a crucial role in cell migration. In this study, the scratch assay was used to determine the involvement of αvβ5 and α5β1 integrin in M14 cell migration. The results showed that the αvβ5 integrin also participates in M14 cell migration. Ruffini *et al*. reported the αvβ5 and αvβ3 integrins to be involved in melanoma cell invasion, while the α5β1 integrin was not (Ruffini et al., 2014). However, my study showed that the high concentration of JBS5 antibody enhanced M14 cell migration. This study showed that treating M14 cells with 2.5 µg/ml of JBS5 antibody stimulated the expression of αvβ3 and induced the ectopic expression of αIIbβ3 on stimulated M14 cells similar to my observations in OSC-19.

4.6 Conclusion

The scratch assay was characterized and then used to evaluate the effect of novel small molecule antagonists on cell migration. OSC-19 was used as a model for multi-integrin expression, DLD-1 was used as a model for αvβ5 integrin, and DU145 and M14 were used as models for αvβ3 and αvβ5. Our findings suggest that the selective inactivation of the α5β1 integrin may not be a safe therapy for head and neck cancer or melanoma due to the activation of intracellular signalling that leads to the stimulation of other sets of integrins to maintain cell migration. Therefore, multi-integrin targeting may be important to inhibit cancer metastasis since it blocks integrins that may be upregulated by

selective α5β1 antagonism. Furthermore, among 10 novel small molecule antagonists, certain compounds were found to have significant antagonistic effects, such as ICT9055 and ICT9072, which were the most effective at reducing DLD-1 cell migration, and ICT9023, ICT9024, and ICT9026, which were the most effective at inhibiting DU145 cell migration. These compounds merit further investigation such as evaluating their effect on cancer cell invasion (using e.g a 3D invasion assay) to assist identification of those compounds being selected for investigation in animal models of tumour invasion and metastasis.

5 CHAPTER 5: General discussion

5.1 Discussion

Integrins are heterodimeric glycoprotein receptors that mediate cell-cell and cell-extracellular matrix interactions. They are involved in several biological processes that are crucial to both body homeostasis and pathological conditions, such as cell proliferation, differentiation, adhesion, invasion, migration, apoptosis and angiogenesis. Moreover, it has been found that integrins are implicated in cancer progression (Goswami, 2013, Ganguly et al., 2013, Seguin et al., 2015). In terms of cancer treatment, the main problems associated with chemotherapy are the long-term toxicities and drug resistance. It is therefore important to identify targets that are highly expressed in tumour tissue but not expressed (or only at low levels) in normal tissue. Immunohistochemistry studies have shown that integrins are overexpressed on tumour tissues when compared to normal tissues (Fabricius et al., 2011, Li et al., 2013, Ha et al., 2014). Further, studies have shown that activated endothelial cells that participate in tumour angiogenesis overexpress integrins αvβ3, αvβ5 and α5β1, whereas quiescent endothelial cells do not (Avraamides et al., 2008, Fabricius et al., 2011, Goodman et al., 2012, Kim et al., 2000a). All these facts have led drug companies to design inhibitors targeting specific integrin-ligand interactions in order to prevent cancer progression and angiogenesis. Many integrin inhibitors have been subject to clinical trials and, while there are 72 trials still active, 46 have been discontinued and only 4 drugs are commercially approved (Goswami, 2013).

Many potential novel small molecule integrin antagonists have been synthesised at the Institute of Cancer Therapeutics and thus need to be evaluated for their potential to block integrin ligation and, consequently, inhibit tumour cell invasion, migration and metastasis. As the tumour cells express several integrins on their surface, one hypothesis suggests the importance of multi-integrin antagonism in order to block entire families of integrins that are involved in cancer progression and angiogenesis.

In this project, the aim was to evaluate the effect of potential novel small molecule integrin antagonists on the inhibition of human tumour cell migration using 2D wound healing assays (scratch assays). This was achieved by:

- Evaluating the targets (αvβ3, αvβ5 and α5β1 integrins) in clinical tumour samples.
- Detecting the expression of αvβ3, αvβ5 and α5β1 integrins in human xenograft tissue as well as in human tumour cell lines.
- Developing models that can be used for both the *in vitro* and *in vivo* evaluation of novel small molecule integrin antagonists.

5.1.1 The expression of αv, β5, αvβ3 and α5β1 integrins in clinical tumour samples

It is very challenging and important when targeting integrins to select the appropriate integrin in a specific type of cancer because the failures of drug evaluation in the clinic could be attributed to incorrect target selection. Failure of cilengitide in head and neck cancer was suggested to be partially due to the

lack of the expression of αvβ3 on tumour cells (Fabricius et al., 2011, Vermorken et al., 2014).

The role of αv, αvβ5, α5β1 and αvβ3 integrin in head and neck cancer has been provided in many reviews (Charalabopoulos et al., 2005, Thomas and Speight, 2001, Ziober et al., 2001). The up regulation of αvβ5 integrin was reported in oral squamous cell carcinoma (Jones et al., 1997), in tongue (Kurokawa et al., 2008) and in laryngeal squamous cell carcinoma (Li et al., 2013). The expression of αvβ5 integrin is associated with lymphatic metastasis and angiogenesis (Li et al., 2013). α5β1 integrin expression was associated with invasion and metastasis, and with a poor prognosis (Shinohara et al., 1999, Vitolo et al., 2000, Charalabopoulos et al., 2005).

The role of αv, αvβ5, α5β1 and αvβ3 integrin in prostate cancer is well documented and was previously reviewed by Goel, Suyin and Sutherland (Goel et al., 2008, Suyin et al., 2013, Sutherland et al., 2012). Studies showed that αvβ3 is implicated in prostate cancer proliferation (De et al., 2003), differentiation (Hurt et al., 2010), adhesion and migration to the bone microenvironment (Cooper et al., 2002). The expression of αvβ5 integrin in prostate cancer cells has been shown to correlate significantly with the Gleason pattern and patient survival (Hess et al., 2014). Moreover, it has been shown that αvβ5 has an important role in the invasion and survival of prostate cancer (Gordon et al., 2009) and in the growth of prostate cancer in bone (Bisanz et al., 2005). In addition, studies have shown that α5β1 integrin is involved in the proliferation of prostate cancer cells (Goel et al., 2010) and adhesion and

spreading of prostate cancer cells interacting with FN (Stachurska et al., 2012). Based on these studies on head and neck and prostate cancer, the expression of αvβ3, αvβ5 and α5β1 was evaluated in clinical prostate and head and neck tumour tissue samples using immunohistochemistry.

The problem faced in such immunohistochemistry studies is the lack of monoclonal antibodies that label integrins in formalin-fixed, paraffin-embedded (FFPE) tissues. Recently, matched rabbit monoclonal antibodies against integrins that are suitable to be used with native and FFPE human tissues have been produced (Goodman et al., 2012). However, these antibodies are not generally available to researchers and, therefore, fresh frozen human tissues were used.

In head and neck cancer tissues, the results show that αvβ3, αvβ5 and α5β1 integrins are extensively expressed in tumour cells but weakly expressed in normal squamous epithelium. Further, our data showed that αv, αvβ3, αvβ5 and α5β1 integrins are more highly expressed in tumour stroma than in the normal stroma, especially αvβ5. The difference between the expression of αv, αvβ3, αvβ5 and α5β1 integrins in tumour tissues and control normal tissues was statistically significant ($P<0.2$ in this very small study). These results are in agreement with the results of Fabricius *et al*., who found that the expression of αvβ5 and α5β1 integrins in tumour tissue was significantly higher than that of squamous epithelium in normal tissues and the expression of αvβ3, α5β1 and αvβ5 was higher in tumour stroma than in normal stroma in control tissue but slightly different regarding αvβ3 expression where they found a low expression

of αvβ3 integrin in the cells of head and neck tumour tissues (Fabricius et al., 2011). In this study, I found a positive correlation between αvβ3, α5β1 and β5 expression and lymph node metastasis.

In the data presented here, among the three integrins, the expression of αvβ3 integrins was very strong in endothelial cells within normal and tumour tissue, in line with a role in cancer angiogenesis as shown in previous studies (Max et al., 1997, Beer et al., 2007). These results indicate that the targeting of αvβ3, αvβ5 and α5β1 integrins affects mainly tumour cells and tumour stroma with little effect on normal tissues. This strategy is important in preventing unwanted inhibition of normal body functions and, consequently, reducing long-term toxicities. It will be necessary to identify an *in vitro* head and neck model which mimics tumour integrin expression to investigate the effect of a combination therapy that targets and blocks αvβ3, αvβ5 and α5β1 integrins in cancer metastasis.

In prostate cancer tissues, the results show that the expression of integrins in tumour cells from high to low was αv, αvβ5, α5β1 and αvβ3, suggesting αvβ3 is not the major αv receptor in prostate tumour cells. The expression of integrins in stroma from high to low was αvβ3, α5β1, αv and αvβ5. The endothelium expressed low level of αvβ3, αvβ5 and αv and did not expressed α5β1.

The limitation associated with this part of the research is the small number of samples (number of head and neck sample = 10 and number of prostate samples = 9) which hampered our efforts to determine the correlation between

integrin expression and clinical outcome. With help from a statistician, Dr Andrew Scally, I was able to find potential correlation by using P value of < 0.2-0.4 as a significant effect. These trends will require confirmation using a larger number of samples.

The results of this study suggest appropriate combination of integrins to target. αvβ3/αvβ5/α5β1 antagonists will be more effective in both head and neck and prostate cancer than single integrin antagonists. In head and neck, αvβ5 and α5β1 antagonists will target tumour and tumour stroma, and to target angiogenesis it is more effective to include αvβ3 integrin in designing the antagonists. In prostate cancer, αvβ3, αvβ5 and α5β1 antagonists will affect tumour and tumour stroma.

5.1.2 The expression of αv, β3, β5 and α5 integrin subunits in a panel of tumour cell lines

Prostate cancer cells PC-3 and DU-145, tongue squamous carcinoma cells OSC-19, and a range of cell lines with either high or low expression of the αv, β5 and α5 integrin subunits; human breast carcinoma cells MCF-7, MCF-7ADR/NCRI ADR-RES cells as a drug-resistant model, melanoma cells M14, colorectal adenocarcinoma cells DLD-1 and HT-29, and human umbilical vein endothelial cells HUVEC based on published results (Goodman et al., 2012, Hirakawa et al., 2006) were used. In order to select cell line models with either high or low expression of the αv, β5 and α5 integrin subunits to use in functional studies, the expression of these integrin subunits was evaluated by Western blot and immunofluorescence. Based on the results obtained from these

evaluations, six cell lines were selected as models to be used to further evaluate the potential of novel small molecule integrin antagonists to inhibit single or multi-integrins *in vitro*. DLD-1 and DU145 express αvβ3, αvβ5 and α5β1 so would be expected to respond best to multi-integrin antagonists, whereas PC-3 and OSC-19 express αvβ5 and α5β1, so would be expected to respond best to dual integrin antagonism of αvβ5 and α5β1. In order to evaluate anti-αvβ5 activity, the cells would need to have αvβ5 integrin as the only RGD-binding integrin and our data showed that MCF-7 and HT-29 express αvβ5 but do not express α5β1, therefore these two cell lines could be used to for this porpose.

5.1.3 The expression of αv, β3, β5 and α5 integrin subunits in a mouse xenograft model

The cell lines identified above could be used to assess integrin functional inhibition *in vitro*. However, they may behave differently *in vivo* because, compared to cell culture, growing human tumour cells *in vivo* is known to alter the expression profile of integrins in response to the extracellular environment (Koshida et al., 2004, Taylor et al., 2012). Therefore, the αv, β3, β5 and α5 integrin subunits expression profile in the corresponding xenografts was evaluated by immunohistochemistry and Western blot and compared to the expression in the corresponding cultured cells. In this study, two rabbit antibodies, Q20 (anti-αv) and ab15459 (anti-β5), were used, both of which showed cytoplasmic immunolabelling in the xenograft tissues. They did not develop any non-specific binding. The αv integrin subunit was expressed in the tumour cells of the HT-29, OSC-19, and MCF-7 xenografts, and in both the

tumour and stromal cells of the PC-3 xenografts. The β5 integrin subunit was expressed in the tumour cells of the HT-29 and MCF-7 xenografts, as well as in both the tumour and stromal cells of the PC-3 xenograft and the stromal cells of the OSC-19 xenograft.

The detection of integrins by mouse antibodies was a challenge in the xenograft mouse tissue because of the development of non-specific bindings. Two mouse antibodies, B7 (anti-β3) and C-9 (anti-α5), were used but I was unfortunately unable to use them to detect the expression of β3 and α5 subunits by immunohistochemistry because of the development of non-specific binding. Western blot results confirmed what was seen in immunohistochemistry; the rabbit antibodies identified the integrin selectively (one band in a specific molecular weight) while the mouse antibodies B7 (anti-β3) and C-9 (anti-α5) gave two bands: one band located at the correct molecular weight (150 kDa and 120 kDa, respectively) and one strong band between 70 and 55 kDa. The expression of the integrin was estimated by excluding the non-specific bands, which were located between 70 and 55 kDa. The Western blot results confirm that the αv, β3, α5, and β5 integrin subunits are expressed in all four xenograft tissues at a variable level, except for the HT-29 xenograft tissue that lacked the α5 integrin subunit.

In general, the expression of αv, β3, β5 and α5 integrin subunits was increased in xenograft tissue compared with the corresponding cultured cells except the expression of β5 and α5 integrin subunits in OSC-19 cultured cells was elevated compared to xenografts. The difference between the expression of

integrins in xenograft tissue compared with the corresponding cultured cells is likely to be due to the contribution of the xenograft stroma in stimulating the expression of integrins. This data is a good starting point to select *in vivo* models to be used once novel antagonists eventually progress to *in vivo* screening.

5.1.4 The effect of integrin antagonists on tumour cell migration

One aim of this study was to evaluate the effect of integrin antagonists on tumour cell migration and the OSC-19 cell line was selected for this purpose because it expresses αv, β5 and α5 integrin subunits.

After optimising the scratch assay conditions, the RGD-binding integrin that is primarily responsible for tumour cell migration was determined by measuring cell migration under the influence of integrin-blocking antibodies. In this study, blocking either the αvβ3 or αvβ5 integrins partially inhibited migration of OSC-19 cells, but blocking the α5β1 integrin enhanced tumour cell migration. These results indicate that a single antagonist is unlikely to completely inhibit tumour migration; therefore, multi-integrin antagonists will be very important in preventing tumour migration (Sheldrake and Patterson, 2014). Antagonist combinations that block αvβ3 and αvβ5 as well as α5β1 and αIIbβ3 integrins significantly inhibited tumour cell migration, which indicates the importance of multi-integrin antagonists in preventing head and neck cancer migration.

Multi-integrin antagonists are expected to have higher efficacy than specific antagonists because of the ability of the integrins to replace one another when one receptor is completely non-functional (Parikka et al., 2006) and by stopping

the crosstalk between integrins that plays a role in many biological processes including the combination or activation of integrins that stimulate or inhibit the function of other integrins in the same cell. Many studies have proven the crosstalk between integrins such as β1, β2, and β3 (Pijuan-Thompson and Gladson, 1997, Simon et al., 1997). For example, the activity of the α2β1 integrin receptor is stimulated by αvβ5/6 integrins in colon cancer (Defilles et al., 2009). Further, inhibition of the β1 integrins in breast cancer enhances the β3 integrin subunits to promote migration or even metastases (Jeanes et al., 2012). Moreover, it has been reported that using antibodies to inhibit the β1 integrin leads to increased expression of β3 through activation of p38 MAPK pathway, which in turn may enhance the metastasis of breast cancer (Parvani et al., 2013). In a future study, it may also be worthwhile to investigate the effects of blocking integrins on cell signalling pathways such as Src and MAPK pathways in order to understand the mechanism that mediates the migration. In the future, other other head and neck cell lines and another anti-α5β1 antibody should be used to confirm our data.

5.1.5 The effect of novel small molecule antagonists on tumour cell migration

The DLD-1 cell line was selected as model to evaluate the effect of multi-integrin antagonism on cell migration due to its expression of αv, β3, β5 and α5 integrin subunits. After the measurement of cell migration under the influence of integrin-blocking antibodies, the results showed that DLD-1 cell migration was mediated primarily by the αvβ5 integrin and partially by αvβ3 and αvβ6. Using this cell line to evaluate the potential of novel small integrin antagonists,

ICT9055 and ICT9072 were identified as blocking cell migration at IC_{20} concentrations. Other compounds did not show any effect at the concentrations used. ICT9055 and ICT9072 have been previously shown to be highly potent αvβ3 antagonists, and ICT9055 was very selective against other RGD-binding integrins in ELISA assays (unpublished results, Mark Sutherland). In order to determine whether the effect of these two compounds on DLD-1 was due to the inhibition of αvβ3 alone or the inhibition of both αvβ3 and αvβ5, the αvβ3 integrin was blocked by preincubating the cells with what was assumed to be a saturation concentration (5 µg/ml) of LM609 antibody. I found that blocking the αvβ3 integrin increased the potential of ICT9055 and ICT9072 to inhibit DLD-1 cell migration. This result suggests that these two compounds have affinity for the αvβ5 integrin as well as αvβ3, and further suggests that the two heterodimers cooperated in the regulation of DLD-1 cell migration. Alternatively this result could indicate that blocking of integrin by either LM609 or compound incompletely inhibits αvβ3 whereas the combination improves receptor occupan.

The role of the αvβ5 integrin in colon cancer is well-documented. As Enns *et al*. (2005) demonstrated, blocking the αv or αvβ5 integrins significantly decreases tumour cell adhesion *in vivo* within the hepatic microvasculature. Yoshioka *et al*. (2010) found that the expression of αvβ5 in LS-LM6 (a highly liver-metastatic human colon cancer cell line) is higher than in the parental cell line (LS174T). Hepatocyte-derived heregulin (HRG) stimulates the αvβ5 integrin to mediate LS-LM6 migration *in vitro*. *In vivo*, they demonstrated that knockdown of αv by small-interfering RNAs (siRNAs) inhibited the liver metastasis of LS-LM6

(Yoshioka et al., 2010). αvβ5 integrin has been shown to suppress the apoptosis of colon carcinoma cells induced by serum deprivation (O'Brien et al., 1996). Data from my study has demonstrated the involvement of the αvβ3 integrin in DLD-1 cell migration, which is in agreement with previous findings concerning its role in colon cancer. For example, a recent study showed that β3 integrin expression was significantly higher in patients with advanced stage, poorly differentiated tumours, and with lymph node invasion than in patients with early stage, well or moderately differentiated tumours, and without lymphatic metastasis (Ibrahim et al., 2013). Integrin antagonists, such as S247, which was described as an αvβ3 antagonist but actually has affinity for all αv integrins, inhibits colon cancer liver metastasis *in vivo* as well as cell proliferation, adhesion and migration, and also mediates apoptosis *in vitro* (Reinmuth et al., 2003). Reinmuth *et al*.'s (2003) study showed that integrins other than αvβ3 and αvβ5 participate in colon cancer metastasis. αvβ6 integrin expression is associated with the tumour's progression and the poor prognosis of patients with colon cancer (Niu et al., 2014). Moreover, αvβ6 regulates colon cancer cell survival through ERK signalling, which results in tumour cell proliferation (Peng et al., 2009). Further, blocking αvβ6 increased the apoptosis of HT-29 cells through decreasing Bcl-2 and increasing Bax, caspase-3 and caspase-9 activity (Zhang et al., 2008). These studies indicate the importance of αvβ6 in colon cancer progression, although our own study showed that the expression of αvβ6 RNA on DLD-1 was low (in house PCR data, Andrew Gordon). Interestingly, in this study, the results of blocking the αvβ6 integrin showed that the lowest concentration of anti-αvβ6 reduced cell migration

whereas the highest concentration had no effect; this may indicate blocking the αvβ6 integrin stimulates the cells to express another integrin or switch on another pathway to mediate cell migration. This corresponds with the findings of Defilles *et al*. (2009) that decreasing the expression of the αv integrins by either siRNA or lysosomal targeting strategies or inhibiting their function using blocking antibodies or disintegrins resulted in enhanced α2β1 integrin-dependent cell migration and increased outside-in signalling as demonstrated by the elevated phosphorylation of FAK and MAPK. This data suggests the importance of multi-integrin antagonists including αvβ3, αvβ5 and possibly αvβ6 in preventing colon cancer metastasis whereas α5β1 had no effect on cell migration.

In order to evaluate the potential of the novel small molecules on prostate cancer, the DU145 cell line was selected as a model to evaluate the effect of multiple integrin antagonism on cell migration since the expression profile showed that this cell line expressed αv, β5 and α5 integrin subunits. Also, our study showed that this cell line expresses αvβ3 but does not express αvβ6 or αvβ8 (in house PCR data, Andrew Gordon). This data is in agreement with the expression profile of RGD-binding integrins in formalin-fixed and paraffin-embedded (FFPE) tissue samples from prostate cancer patients where the immunohistochemistry results showed that the αvβ6 and αvβ8 integrins were not expressed in tumour cells, and that αvβ3 was expressed in the blood vessels only. The αvβ5 integrin was almost always expressed in prostate cancer cells in correlation with the Gleason pattern (GP) (Hess et al., 2014,

Goodman et al., 2012). Based on this data, the involvement of either the αvβ6 or αvβ8 integrins in DU145 cell migration was thought unlikely.

The measurement of cell migration under the influence of integrin-blocking antibodies indicated that both the αvβ5 and αvβ3 integrins play a major role in DU145 cell migration, whereas the α5β1 integrin has a reverse effect whereby the lowest concentration of α5β1 antibody significantly reduced cell migration while the highest concentration did not affect cell migration. Again, this could be because the complete inhibition of a particular integrin activates intracellular signalling to mediate cell functions by other integrins; if α5β1 is not able to signal, the intracellular signalling effectors are redirected to other integrins, thus enhancing their signalling. This was demonstrated by Kim who found the α5β1 integrin to regulate the αvβ3 integrin and mediate endothelial cell migration and angiogenesis(Kim et al., 2000b). Similarly, Lishko *et al*. identified that increasing the levels of integrin αMβ2 retards α5β1-mediated migration (Lishko et al., 2003). α5β1 integrin mediates the adhesion of prostate cancer cells to fibronectin (Witkowski et al., 1993). Stachurska *et al*. found that blocking only one of either α5β1 or αvβ3 did not completely inhibit the cell adhesion to fibronectin, indicating the importance of inhibiting all redundant integrins to affect cell functions (Stachurska et al., 2012).

Evaluation of the impact of the novel compounds on DU145 cell migration showed that ICT9023, ICT9024, and ICT9026 had no cytotoxicity at 50 µM and had a high effect in inhibiting DU145 cell migration. ICT9085, ICT9094,

ICT9019, ICT9087, ICT9073, and ICT9055 had a moderate effect on cell migration (about 20%).

These result encourages the investigation of the effects of ICT9023, ICT9024, ICT9026 ICT9055 and ICT9072, compounds on other functional assays, such as the 3D Boyden Chamber transwell migration assay, to evaluate the effect of integrin inhibitors on tumour cell invasion. As part of the compound development process, it would be useful to identify the effects of these compounds on cell signalling pathways as potential biomarkers of efficacy. Moreover, the pharmacokinetic and pharmacodynamics of the most active compounds should be estimated before starting *in vivo* studies.

5.2 Conclusion

This study has evaluated the expression at the protein level of a panel of integrins in cell culture, xenografts and in clinical material for head and neck, prostate and colon cancers. The results support the potential for the use of integrin inhibitors, particularly multi-integrin inhibitors, to prevent cancer cell migration, a prerequisite for tumour invasion and metastasis. By using an *in vitro* 2-D migration assay (scratch assay), novel small molecule integrin antagonists were shown to have significant antagonistic effects on tumour cell migration, at concentrations considerably lower than required to show cytotoxicity, suggesting that these compounds could be further developed for use in chronic inhibition of tumour invasion and metastasis.

5.2.1 Future work

- Evaluate the expression of αv, β5, αvβ3 and α5β1 integrins in a larger clinical sample size to determine the correlation between integrin expression and cancer progression.

- Confirm the role of α5β1 integrin in head and neck cell migration by using another α5β1 integrin antibody and other head and neck cancer cell lines.

- Determine the effect of blocking α5β1 integrin on the signalling pathways, especially the P38 MAPK pathway.

- Use proteomics to identify alterations in protein expression that could influence cell migration after blocking α5β1 integrin; this study will help to design selective compounds for these specific proteins.

- Investigate the effect of novel small molecule integrin antagonists on tumour cell invasion by using a further 2D assay, such as the modified Boyden chamber assay.

- Use of tumour spheroids as a 3D invasion model to study the effect of novel small molecule integrin antagonists.

- Determine the effect of novel small molecule integrin antagonists on signalling pathways.

- Estimate the pharmacokinetics and pharmacodynamics of the most active compounds *in vivo*.

6 CHAPTER 6: References

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7 appendix

PUBLICATIONS

Conference abstracts and proceedings

Hanadi Ahmedah, Laurence Patterson, Steven D Shnyder, Helen M Sheldrake. “INVESTIGATION OF THE EXPRESSION AND THE ROLE OF αvβ3, αvβ5 and α5β1 INTEGRINS IN HEAD AND NECK CANCERS”. EACR-23, the 23rd Biennial Congress of the Association for Cancer Research, 5 - 8 July 2014, Munich, Germany.

Hanadi Ahmedah, Laurence Patterson, Steven D Shnyder, Helen M Sheldrake. “INVESTIGATION OF THE EXPRESSION AND THE ROLE OF αvβ3, αvβ5 and α5β1 INTEGRINS IN COLON CANCER”. The Eighth Annual Saudi Student Conference, January 31 to February 1 2015, London, UK.